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Formation of L-Glutamic Acid from γ-Aminobutyric Acid by Plant Enzyme

Part II. On Fractionation of α-Ketoglutaric-γ-aminobutyric Transaminase and L-Glutamic Acid Decarboxylase in Plant Enzyme Preparation

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Received January 28, 1958

In Part 1 of this series, the present authors have reported that α-ketoglutaric-γ-aminobutyric transaminase may be present in the L-glutamic acid decarboxylase preparation.

After repeating experiments, it has recently been confirmed that the L-glutamic acid decarboxylase preparation reported by Okunuki and Schales et al. contains two kinds of enzymes, α-ketoglutaric-γ-amino-butyric transaminase and L-glutamic acid decarboxylase.

INTRODUCTION

Plant L-glutamic acid decarboxylase was first discovered by Okunuki,$^{1)}$ and the enzyme preparation isolated by Okunuki et al.$^{2)}$ in 1951. The property of the enzyme preparation was investigated by Martin$^{3)}$ with ^{14}COOH-labeled L-glutamic acid, and it was reported in 1954 that the enzyme preparation catalyses the decomposition of L-glutamic acid to γ-aminobutyric acid plus CO_2, almost quantitatively.

Thence, through our studies$^{4)}$ on the distribution of L-glutamic acid decarboxylase in edible plants and the reverse action of the enzyme, it has been made clear that α-keto-glutaric-γ-aminobutyric transaminase may be demonstrated in the same plant tissue containing L-glutamic acid decarboxylase, and finally, the occurrence of α-ketoglutamic-γ-aminobutyric transaminase activity was confirmed by our collaborators in the L-glutamic acid decarboxylase preparation.

Recently, research was extended to the fractionation of α-ketoglutaric-γ-aminobutyric transaminase and L-glutamic acid decarboxylase in plant enzyme preparations. At first, experiments were repeated for the preparation of L-glutamic acid decarboxylase and α-ketoglutaric-γ-aminobutyric transaminase from beet root and squash pulp, and enzymic activities of the Preparations obtained were tested.

In later experiments, the fractionation of the crude enzyme was carried out by partial precipitation with ammonium sulfate, and each fraction obtained was repeatedly tested for the α-ketoglutaric-γ-aminobutyric transaminase and L-glutamic acid decarboxylase activities. Marked activities of both enzymes were recognized in squash rind, but these activities were extremely low in the pulp. The transaminase activity seemed to assume its maximum in the fraction at 0.3 saturation of ammonium sulfate, and the decarboxylase action was located in the fraction at 0.5 saturation.

Finally, the glutamic acid formed by this

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1) K. Okunuki, *Botan. Mag.* (Tokyo) **51**, 270 (1937).

2) K. Okunuki, et al., *Proc. Japan. Acad.*, **27**, 658 (1951).

3) E. H. Martin et al., Division of Biological Chemistry. C & EN (1954).

4) T. Suzuki, et al., This Bulletin, **22**, 39 (1958).

transaminase was confirmed to be of the L-from configuration by paper partition chromatography and by paper electrophoresis.

No positive results was obtained to indicate that L-glutamic acid may be formed from α-ketoglutaric acid and ammonium salts by this enzyme.

From the experimental results, it seems justifiable to conclude that the well-known preparation of the L-glutamic acid decarboxylase reported by Okunuki and Schales may be composed of two kinds of enzymes i.e., α-ketoglutaric-γ-aminobutyric transaminase and L-glutamic acid decarboxylase. Further research for complete purification of both enzymes is now in progress.

EXPERIMENTAL

1. Occurrence of α-Ketoglutaric-γ-aminobutyric Transaminase and L-Glutamic Acid Decarboxylase in Beet Root Juice.

The enzyme preparations were obtained from the clear juice of beet root according to Scheme 2 in Part I of the previous paper[5], yielding 220 mg/100 g raw material, and enzymic activities were tested under the same condition as in the case of squash rind enzymes.

A portion of 0.5 g of α-ketoglutaric-γ-aminobutyric transaminase preparation was dissolved in 10 ml of M/15 phosphate buffer solution of pH 7.4, to which 103 mg of γ-aminobutyric acid and 73 mg of α-ketoglutaric acid were added; after incubation for 24 hrs at 37°C, the L-glutamic acid formed was estimated by paper chromatography, using Toyo filter paper No. 50, and a solvent of acetic acid : *n*-butanol : water (1 : 4 : 2), and also by ninhidrin coloration phtometrically, using an Erma Type II-A photometer, filter-57. (Table I, II)

2. Activity of α-Ketoglutaric-γ-aminobutyric Transaminase Preparation obtained from Squash Pulp.

Fresh clear juice was first prepared from 100 g of squash pulp and the fluid adjusted to pH 5.8 or 8.4 with M/15 phosphate buffer, and the transaminase preparation was isolated by the process as mentioned in Scheme 3 of the previous paper[5]. The activity of the enzyme was tested by paper chromatography and the ninhidrin coloration described above, and the amount of L-glutamic acid formed was determined as shown in Table III.

3. Fractionation of the Enzyme Preparations.

Fractionation of the enzyme preparations in squash juice was carried out by partial precipitation with ammonium sulfate according to Iwamoto, Hayashi and Sahashi[6]. The first stage of fractionation was carried out 0.1, 0.3, 0.5, 0.8, and 1.0 saturations of ammonium sulfate; the enzymic activities of each fraction thus obtained were accurately investigated for both enzymes as described above. (Table IV, Figs. 1, 2)

TABLE I. FORMATION OF L-GLUTAMIC ACID FROM γ-AMINOBUTYRIC ACID AND α-KETOGLUTARIC ACID BY PLANT α-KETOGLUTARIC-γ-AMINOBUTYRIC TRANSAMINASE PREPARATION OBTAINED FROM BEET ROOT JUICE.

Total L-Glutamic Acid in the Reaction Mixture	L-Glutamic Acid in the Enzyme Preparation	L-Glutamic Acid formed	L-Glutamic Acid/ γ-Aminobutyric Acid
1120 μg/10 ml	0	1120 μg/10 ml	1.08%

TABLE II. ACTIVITY OF L-GLUTAMIC ACID DECARBOXYLASE PREPARATION OBTAINED FROM BEET ROOT.

No. of Exp.	Substrate (L-Glutamic Acid)	Enzyme Preparation	CO_2 liberated* for 50 min.	CO_2
1	0.5 mg	Beet Root 4 mg	68.83 μl	90.4%
2	0.5	Squash Rind 4 mg	69.65	91.6
Calcd.	0.5		76.15	100.0

* The Liberation of CO_2 from pure L-glutamic acid was determined by the Warburg apparatus.

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[5] T. Suzuki, et al., This Bulletin, **22**, 41 (1958).

[6] K. Iwamoto, J. Hayashi, Y. Sahashi, *J. Vitaminology*, **3**, 238 (1957).

TABLE III. FORMATION OF L-GLUTAMIC ACID FROM γ-AMINOBUTYRIC ACID WITH α-KETOGLUTARIC ACID BY PLANT α-KETOGLUTARIC-γ-AMINOBUTYRIC TRANSAMINASE PREPARATION OBTAINED FROM SQUASH EDIBLES.

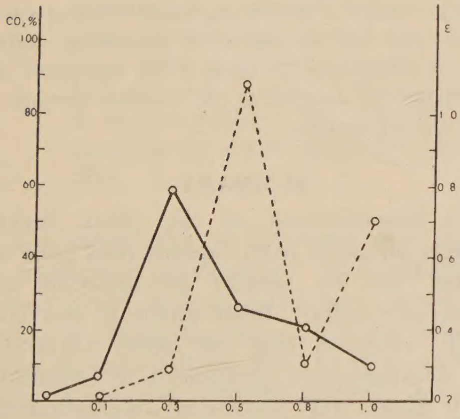
L-Glutamic acid in the reaction mixture	L-Glutamic acid in the enzyme preparation	L-Glutamic Acid formed	L-Glutamic acid/ γ-Aminobutyric acid
3500 μg/10 ml	0	3500 μg/10 ml	3.39%

TABLE IV. AMOUNT OF THE ENZYME PREPARATIONS IN EACH FRACTION OBTAINED FROM 100 G OF RAW MATERIAL.*

Saturation $(NH_4)_2SO_4$	Enzyme preparation obtained from squash rind	Enzyme preparation obtained from squash edible pulp
0.1	53 mg	12 mg
0.3	1550	180
0.5	2000	840
0.8	2360	880
1.0	4550	2200

* The preparations were obtained from Raw Material by Scheme 3.

Raw Material Homogenate (100 g)
↓ added M/15 phosphate buffer pH 7.4
Incubation for 40 min. at 37°C
↓ filtered with lint centrifuged for 20 min. at 3000 r.p.m.
Transparent Fluid
↓ added ammonium sulfate centrifuged for 20 min. at 3000~8000 r.p.m.
Precipitate
↓ dried over $CaCl_2$ in a vacuum desicator
Enzyme preparation



1. FIG. Distribution of the Enzyme Activities in Each Fraction of the Enzyme Preparation obtained from Squash Rind.

—— Transaminase; ---- Decarboxylase; CO_2%, Percentage of CO_2 liberated from pure L-glutamic acid by decarboxylation; E, value of ninhidrin coloration of L-glutamic acid formed by transamination.

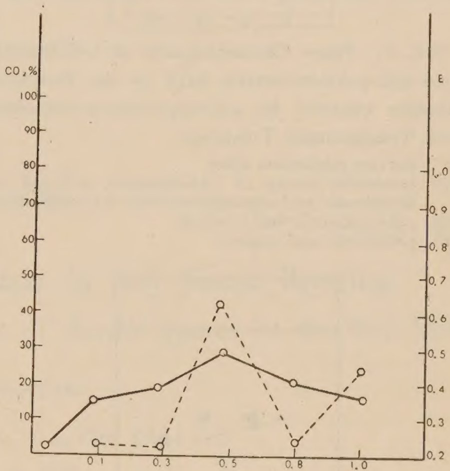


FIG. 2. Distribution of the Enzyme Activities in Each Fraction of the Enzyme Preparation obtained from Squash Edibles.

—— Transaminase; ---- Decarboxylase; CO_2˙, percentage of CO_2 liberated from pure L-glutamic acid by decarboxylation; E, value of ninhidrin coloration of L-glutamic acid formed by transamination.

4. Stereochemical Identification by Paper Chromatography of L-Glutamic Acid formed by α-Ketoglutaric-γ-aminobutyric Transaminase.

Five hundred mg of α-ketoglutaric-γ-aminobutyric transaminase preparation was dissolved in 10 ml of M/15 phosphate buffer solution of pH 7.4 to which 103 mg of γ-aminobutyric acid and 73 mg of α-ketoglutaric acid were added. After incubation for 24 hrs at 37°C, the L-glutamic acid formed was estimated by paper chromatography and ninhidrin colorimetry. (Fig. 3)

On the other hand, when the reaction mixture was heated at 100°C, and the resultant substance was again treated by L-glutamic acid decarboxylase for one hr, at pH 5.8 and at 37°C, the product obtained did not show the presence of the L-glutamic acid by paper partition chromatography and ninhidrin coloration, as seen in Fig. 4.

5. Stereochemical Identification by Paper Electrophoresis of L-Glutamic Acid formed by

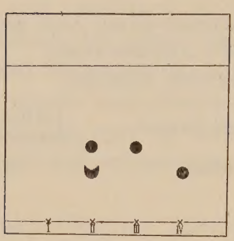


FIG. 3. Paper Chromatograms of L-Glutamic Acid and γ-Aminobutyric Acid in the Reaction Mixture obtained by α-Ketoglutaric-γ-aminobutyric Transaminase Treatment.

(I) Enzyme preparation alone
(II) Incubation mixture of γ-aminobutyric acid and α-ketoglutaric acid administrated with the transaminase
(III) γ-Aminobutyric acid (control)
(IV) L-Glutamic acid (control)

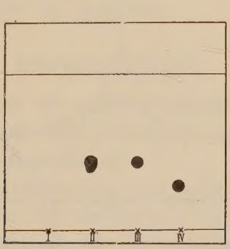


FIG. 4. Paper Chromatograms of the Reaction Mixture after Treatment of L-Glutamic acid Decarboxylase, indicating the Disappearance of the Spot of L-Glutamic Acid.

(I) Enzyme preparation alone
(II) Reaction mixture after the treatment of L-glutamic acid decarboxylase, indicating the disappearance of the spot of L-glutamic acid.
(III) γ-Aminobutyric acid (control)
(IV) L-Glutamic acid (control)

α-Ketoglutaric-γ-aminobutyric Transaminase.

Electrophoretic separation of the reaction mixture obtained by α-ketoglutaric-γ-aminobutyric transaminase treatment was carried out after thermal decomposition under the following conditions, McIlvaine's buffer[7] pH 5.8, 2 mA, 400～250 V, and 3 hrs. L-glutamic acid moved towards the cathode during electrophoresis, whereas γ-aminobutyric acid moved towards the anode as shown in Fig. 5.

In the electrophoretic experiments before the thermal decomposition of the reaction mixture, no L-glutamic acid was detected hence it was realized that L-glutamic acid formed may always be degraded into γ-aminobutyric acid during electrophoresis by the L-glutamic acid decarboxylase contained as an impurity. (Fig. 6.)

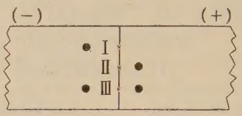


FIG. 5. Paper Electrophoretic Pattern of L-Glutamic Acid and γ-Aminobutyric Acid in the Reaction Mixture after Thermal Decomposition of the Enzyme.

(I) γ-Aminobutyric acid (control)
(II) L-Glutamic acid (control)
(III) Reaction mixture, indicating the Spots of γ-aminobutyric acid and L-glutamic acid.

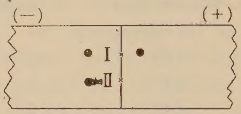


FIG. 6. Paper Electrophoretic Pattern of L-Glutamic Acid and γ-Aminobutyric Acid in the Reaction Mixture before Thermal Decomposition of the Enzyme.

(I) γ-Aminobutyric acid and L-glutamic acid (standard mixture)
(II) Reaction mixture before thermal decomposition, indicating the disappearance of L-Glutamic acid.

6. None-Formation of L-Glutamic Acid from α-Ketoglutaric Acid and Ammonium Sulfate.

The transaminase preparation obtained here was again tested for ability of the L-glutamic acid formation from α-ketoglutaric acid and ammonium salts. To 500 mg of the enzyme preparation dissolved in 10 ml of M/15 phosphate buffer of pH 7.4 was added 73 mg of α-ketoglutaric acid and an excess of ammonium sulfate. After incubation for 24 hrs at 37°C, L-glutamic acid could never be detected in the reaction mixture by paper chromatography.

SUMMARY

(1) Fractionation of the plant enzyme proteins obtained from Squash rind juice was carried out by partial precipitation with ammonium sulfate, from which it was completely revealed that the plant L-glutamic acid decarboxylase preparation as reported in many papers may consist of α-ketoglutaric-γ-aminobutyric transaminase and L-glutamic acid decarboxylase.

(2) Further stereochemical evidence of L-glutamic acid was obtained by paper partition chromatography and by paper electrophoresis,

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[7] T. C. McIlvaine, *J. Biol. Chem.*, **49**, 183 (1921).

and the L-glutamic acid formed by this transaminase was proved to be of the L-form configuration.

(3) The preparation containing transaminase obtained here was tested for its activity of L-glutamic acid formation from α-ketoglutaric acid and ammonium sulfate, and always gave negative results.

The expence of this research was partly defrayed by a grant in acid from the Ajinomoto Co., Inc., to which the authors' thanks are due. The authors also wish to thank Messrs. T. Miki, N. Tsukada, M. Matsumoto of this laboratory for their generous assistance.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 6, p. 345～352, 1958]

Studies on the Activities of Bacteria in Soy Sauce Brewing

Part II. The Proteinases and the Existence of *Bacilli* Spores in the Soy Mash

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Received March 10, 1958

The activity of proteinases in soy masy juice was measured by a modification of the Anson's method[1]. Compared to proteinases of purely cultured soy koji digest which consisted of only *Aspergillus* proteinases, no difference was observed with the proteinases of usual soy mash through the studies of pH-proteinese activity curves, the inhibition test, and inactivation by acid and alkali treatment. It is supposed, therefore, that the proteinases in soy mash are mostly of *Aspergillus* origin. Viable counts on salted media, heat shocking at 80°C for thirty minutes, and the microscopic counts proved that there are very few *Bacilli* vegetative cells in soy mash. From these facts the author assumed that *Bacilli* mainly grow in the koji and survive in the soy mash mostly in the form of spores. This work also suggested that the so-called "*Aspergillus* alkaline proteinase" consists of two distinct proteinases, and further the existence of an inhibitor which only acts at pH 8.

The preparation of soy sauce is as follows; A mixture of steamed soy bean and parched wheat grain is inoculated with spores of a strain of *Aspergillus sojae*[2], and the inoculated mass incubated from 25°C to 40°C untill the mass is covered with the mycelia and spores of *Asp. sojae.* The soy koji thus prepared is soaked in heavy brine of crude salt and the final concentration of sodium chloride is brought up to about 18%. In the mash, proteolysis, starch hydrolysis, fermentation by osmophilic bacteria and yeasts[3] take place and lost during one year's brewing in room temperature. The main organic components of soy sauce are amino acids[4], small peptides[5], organic acids[5], alcohols, sugars, phenolic substances[6], etc.. An important characteristic of soy sauce brewing is that the main microbial action is proteolysis.

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[1] M. L. Anson, *J. Gen. Physiol.*, **22**, 79 (1938).

[2] K. Sakaguchi and Y. Yamada, *J. Agr. Chem. Soc. Japan*, **20**, 64, 141 (1944).

[3] H. Onishi, This Bulletin, **21**, 151 (1957).

[4] T. Tsunoda, et al., *J. Agr. Chem. Soc. Japan*, **26**, 477 (1952).

[5] S. Hori et al., *ibid.*, **30**, 519 (1956); **31**, 17, 19, 22 (1957).

[6] T. Yokotsuka, *ibid.*, **23**, 200 (1949), **24**, 355, 358, 402, 407 (1951), **25**, 206, 401, 446 (1952), **27**, 276, 334, 359, 549 (1953).

Matsumoto⁷⁾ isolated various kinds of *Bacilli* strains from soy mash. Ishimaru⁸⁾ reported on many species of salt tolerant *Bacilli*, *Pediococci*, and a few strains of *Micrococci* in soy mash. It is a well known fact that aerobic bacilli has quite a strong proteolytic activities. It has, therefore, been assumed that the proteolysis which occurrs in soy sauce brewing would be due to the proteinases produced by the koji mold, *Aspergillus*, and to those produced by other salt tolerant bacteria, especially, *Bacillus*. In order to confirm this supposition, the two series of experiments were conducted.

(1) The comparison of properties of proteinases in soy mash with those of proteinases of soy koji digest made aseptically.

(2) A quantitative estimation of the number of aerobic bacilli surviving in soy mash.

Some results obtained from these experiments will be described in this paper.

MATERIALS AND METHODS

Enzyme Solutions:

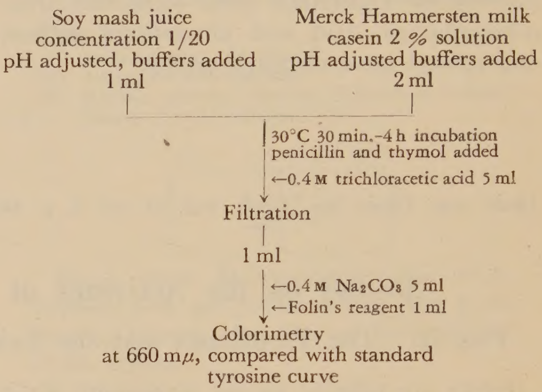
Factory Soy Mash Juice. The soy mash which was aged for various months for ripening was supplied by the Seventh Factory of Noda Shoyu Co. Ltd.. The juice of this factory soy mash which was diluted twenty- or forty-fold was used as the enzyme solution of proteinases in soy mash.

Purely Cultured Soy Koji Digest⁹⁾. As a control to the factory soy mash, purely cultured soy koji digest was prepared in the following way. Steamed soy bean and parched wheat grain were sterilized in two necked large Komagome flasks⁹⁾, inoculated with *Aspergillus sojae* strain KS, and incubated for three days at 30°C. The koji thus prepared, is hereafter named as purely cultured soy koji. This soy koji was aseptically soaked in about an equivalent volume of brine, and the final salt concentration was brought up to 18%. After one year of maturing, four flasks containing 900 ml of the soy koji digest were obtained. The juice was used as the enzyme solution in the same manner as factory soy mash.

Substrate for Proteinase. A 2% solution of glass-bottled Hammersten casein (Merck Co.) was used.

Proteinase Assay. The method is shown on Table I.

TABLE I. A MODIFICATION OF ANSON'S METHOD FOR THE ASSAY OF SOY MASH PROTEINASE


Soy mash juice
concentration 1/20
pH adjusted, buffers added
1 ml
Merck Hammersten milk
casein 2 % solution
pH adjusted buffers added
2 ml
30°C 30 min.–4 h incubation
penicillin and thymol added
←0.4 M trichloracetic acid 5 ml
Filtration
1 ml
←0.4 M Na₂CO₃ 5 ml
←Folin's reagent 1 ml
Colorimetry
at 660 mμ, compared with standard
tyrosine curve

The difference from the control was offered as the proteinase activity. As the control, the substrate solution was substituted to buffers and incubated in the same way.

The activity was presented by mg tyrosine per ml of the original soy mash juice.

For the pH adjustment, Beckman Model G pH meter was used. On alkaline side the Alkaline Glass Electrode was recommendable.

Viable Count of Bacteria. One ml of the soy mash was added to 49 ml of sterilized 17% salt broth, and 0.5 ml of the diluted mash solution was transferred aseptically to the next dilution. From the twice or thrice diluted solution, 0.5 ml were taken and plated out in the agar medium. Difco's heart infusion agar supplemented with 1% glucose and 0.5% dipotassium phosphate was used as the plating medium. In order to prevent the growth of fungi and yeasts which is abundant in soy mash, eurocidin¹⁰⁾ was added to the plating medium in a concentration of 30 γ/ml. When the concentration of sodium chloride was 6 % or more, the eurocidin concentration could be reduced to 10γ/ml.

pH-activity Curves of the Soy Mash Proteinase. To control the hydrogen ion concentration, the acid-alkali titration curves of both 2 % casein solution and enzyme solution were prepared using a Beckman Model G pH meter. The necessary amounts of acid or alkali were previously added to casein solution to attain the desired pH value after the addition of enzyme solution. The buffer solutions were; Walpole's acetate buffer

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7) K. Matsumoto, *J. Soc. Brewing Japan*, **21**, (1926), **22**, (1927), **23**, (1928), **24**, (1929).

8) Y. Ishimaru, *J. Agr. Chem. Soc. Japan*, **9**, 859, 953, 1143 (1933).

9) K. Sakaguchi, This Bulletin, in press. (Part V)

10) K. Sakaguchi, *J. Agr. Chem. Soc. Japan*, **29**, 758 (1954); *Rep. Noda Inst. Sci. Res.*, **2** (1958).

from pH 2.0 to 4.0, Sörensen's phosphate buffer from pH 5.0 to 8.0, Atkins and Pantin's borate buffer at pH 9.0 and 10.0, Kolthoff's phosphate buffer at 11.0 and 12.0 and Sörensen's glycine buffer at pH 13.0. The ionic strength was one half of each standard buffer solution.

In some experiments, salt was added to all the enzyme samples to bring up the practical concentration of soy mash to 17 %.

Detection of the Difference between the Proteinases of the soy Mash and the Purely Cultured Soy Koji Digest with the Aid of Specific Inhibitors against *Aspergillus* Proteinases. It has been known that proteinases produced by *Aspergillus* consist of at least three proteinases; namely, Acid Proteinase[11,12], Neutral Proteinase[13], and Alkaline Proteinase[14,15,16]. The effects of specific inhibitors against these three *Aspergillus* proteinases were examined. The inhibitors used were; sodium laurylsulphonate against Acid Proteinase[13], ethylene-diamine-tetraacetic acid and hydrogen peroxide against Neutral Proteinase, potato inhibitor[17] and mercuric acetate against Alkaline Proteinase. Preincubation time with inhibitors required, was thirty minutes.

Detection of the Difference between the Proteinases of the Soy Mash and the Purely Cultured Soy Koji Digest by Acid and Alkali Treatment. Samples were identical with former experiments. As for the acid treatment, the enzyme solution was kept at pH 3.0 and 37C° for one hour. In case of alkali treatment the solution was kept at pH 9.0 and 37C° for twenty-four hours.

RESULTS

1. pH-activity Curves of Proteinase of Fresh and Matured Soy Mash. Fresh and matured soy mash juices, namely one month and twelve months old, were sampled from both factory mash and purely cultured soy koji digest, of which pH-activity curves were determined (Fig. 1). The curves obtained revealed some interesting facts. Firstly, factory soy mash proteinase showed identical types of curves with those of the fresh and matured soy koji digest which contained only *Aspergillus* proteinases, and no special peak due to bacterial proteinase was observed. The next distinctive character was a trough at pH 8.0, and, with the exception of this trough, the curves showed no difference from the pH-activity curve of *Asp. sojae* proteinase produced in wheat bran culture[18]. This inactivation of *Asp. sojae* proteinase at pH 8.0 will be referred to later.

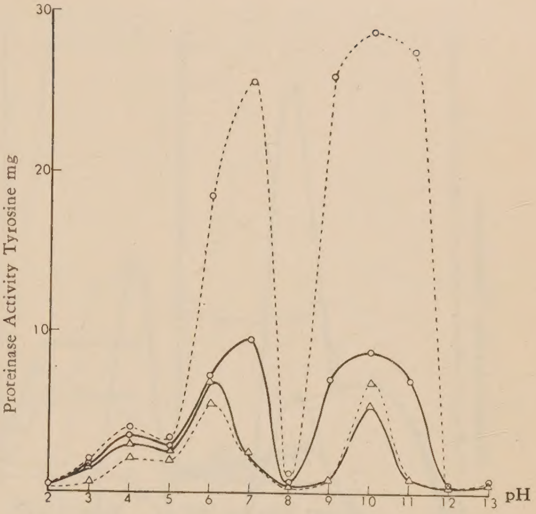

FIG. 1. pH-Proteinase Activity Curves of Purely Cultred Soy Koji Digest and Factory Soy Mash.

---○--- 1 month
---△--- 12 months } purely cultured soy koji digest
——○—— 1 month
——△—— 12 months } factory soy mash

A considerable difference in proteinase activity between purely cultured mash and factory one-month mash seems to be due to the inactivation of proteinases by the rapid pH depression to about 4.4 in the case of factory soy mash, which is probably caused by the growth of soy sauce lactic acid bacteria, *Pediococci*[9,19].

2. pH-activity Curves of Soy Mash Proteinases in the 17 % Salt Solution. The two enzyme origins used were; factory soy mash brewed

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11) K. Kageyama and O. Sugita, *J. Ferment. Technol. Japan*, **33**, 109 (1955).
12) F. Yoshida and M. Nagasawa, This Bulletin, **20**, 252, 257, 262 (1956).
13) T. Yasui, This Bulletin, in press.
14) T. Mizunuma and N. Iguchi, This Bulletin, **22**, 35 (1958).
15) W. G. Crewther et al., *Austr. J. Biol. Sci.*, **6**, 410 (1953).
16) K. Wallenfels, *Biochem. Z.*, **321**, 189 (1950).
17) K. Matsushima, *J. Agr. Chem. Soc. Japan*, **29**, 883 (1955).
18) N. Iguchi and K. Yamamoto, ibid., **29**, 387, 394 (1955).
19) K. Sakaguchi, This Bulletin, in press. (Part III, IV, V)

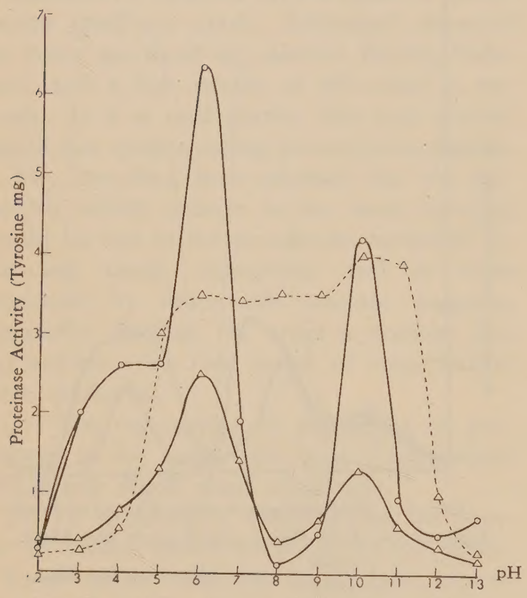

FIG. 2. pH-Proteinase Activity Curves of Soy Mash in 17 % NaCl Solution.

—○— In M/30 buffer solution

—△— In the same buffer solution containing 17 % NaCl

---△--- Juice of purely cultured soy koji digest. Strain X-816 was used for the preparation of the koji. In the same buffer solution containing 17 % NaCl.

for one year and a one year-old purely cultured soy koji digest. The purely cultured soy koji was prepared with *Asp. sojae* mutant X-816 which has a stronger proteolytic activity than *Asp. sojae* strain KS. As shown in Fig. 2, in the environment of 17 % salt solution, the pH curve of the soy mash proteinases declined to one-half or one-third in height of the activity in a salt-free environment, showing an identical type of curves, revealing no special peak.

The pure cultured soy koji digest of X-816 strain had higher proteolytic activities even after one year's digestion. Moreover, it showed no trough at pH 8.0, and, this was also observed in the early stage of digestion. This fact suggested the absence of the specific inhibitor acting at pH 8.

3. The Difference between the Proteinases of the Purely Cultured Soy Koji Digest and those of the Soy Mash by the use of Specific Inhibitors and Acid and Alkali Treatment. As shown in Table II, the difference between the proteinases of factory soy mash and purely cultured soy koji digest was hardly recognizable except some differences observed in activities at pH 4.0 with EDTA and mercuric acetate. The reason for these differences was not clear, because in other experiments, the inhibition of factory soy mash proteinase by the two inhibitors at pH 4.0 was not observed. The proteinases of factory soy mash should, therefore, considered to be almost of *Aspergillus* origin, and it may be added that the detection of bacterial proteinase remained unsuccessful. Fig. 3 also shows the absence of any special proteinase which is resistant to the acid and alkali treatment.

4. "Inhibitor acting at pH 8.0" in the Soy Mash Juice. The occurrence of a deep trough at pH 8.0 in proteinase pH curves is peculiar to the soy mash and the purely cultured soy koji digest, since this is not discernible on the soy koji extract proteinase[18]. It is conceivable

TABLE II. THE INHIBITION RATIO (%) OF SOY MASH PROTEINASES BY SPECIFIC INHIBITORS AGAINST THREE *Aspergillus* PROTEINASES

Incubation pH	pH 4.0				pH 6.0				pH 10.0			
Duration of brewing in months	12		1		12		1		12		1	
Culture	Pure	Factory	Pure	Factory	Pure	Factory	Pure	Factory	Pure	Factory	Pure	Factory
Potato inhibitor	11	4	7	8	51	15	65	40	85	91	81	85
Hg-acetate	3	30	−6	21	34	51	26	26	13	46	31	41
H_2O_2	4	3	−21	−2	−5	−29	−2	−35	−13	−7	−8	−2
E.D.T.A.	3	39	3	44	37	34	18	46	3	8	1	3
S.L.S.	62	42	47	47	9	5	7	5	−5	−1	8	12

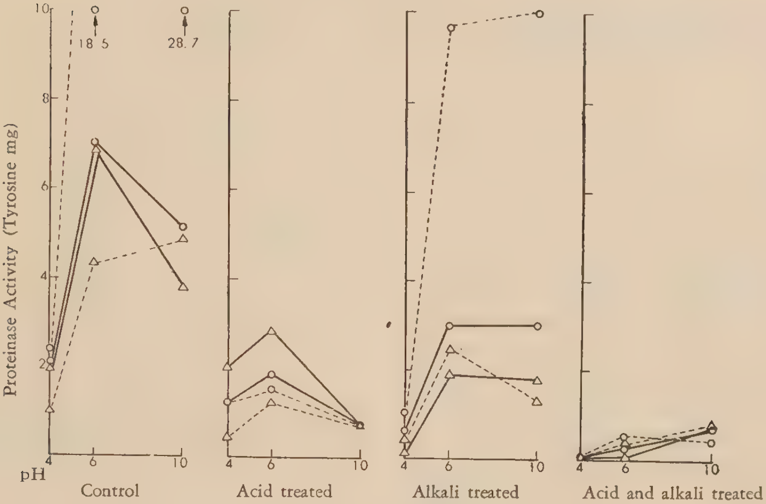

FIG. 3. Acid and Alkali Treated Soy Mash Proteinase.

---○--- 1 month
---△--- 12 months } purely cultured soy koji digest
——○—— 1 month
——△—— 12 months } factory soy masy

that this phenomenon is caused by an inhibitor in the soy mash juice which reacts with *Aspergillus* proteinase, specifically at pH 8. The following experiments were conducted in order to confirm this supposition.

The extract of molded bran prepared with *Aspergillus sojae* strain KS. was properly diluted and used as the enzyme solution. To the enzyme solution, was added an equivalent volume of the ten-fold diluted solution of purely cultured soy koji digest as the inhibitor. The mixture was kept overnight in the refrigerator and then proteinase activity at pH 8.0 was measured. In parallel, the extract of molded bran was added with salt to the concentration of 17 %, pH adjusted at 4.7, and kept for one week in the refrigerator to examine the effect of low pH and high salt concentration. The effect of the soy bean trypsin inhibitor[20] was also examined (Table III).

The inactivation ratio of soy koji digest juice was far beyond the other two conditions, suggesting the existence of a strong inhibitor in the soy koji digest juice.

Occasionally, the juice was stored in the refrigerator for two months and a white protein-like precipitation appeared. Measurement of the proteinase activity in the supernatant showed that a high proteolytic activity is retained. On the other hand, when the precipitates were re-dissolved in the diluted

TABLE III. AN INHIBITOR OF SOY MASH ACTING AT PH 8

Inhibitor	Proteinase activity
Control	100%
NaCl 18%, pH 4.7	86
Soy koji digest juice, concentration 1/10, 1 : 1	64
Soy bean trypsin inhibitor	104

TABLE IV. THE INHIBITORY ACTION OF SOY MASH JUICE PRECIPITATE PRODUCED BY TWO MONTHS REFRIGERATION

Enzyme solution	pH	Proteinase activity
Supernatant	6.0	100 %
	8.0	110
Precipitate was dissolved	8.0	47

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20) M. Kunitz, *J. Gen. Physiol.*, **29**, 149 (1947), **30**, 291 (1947).

TABLE V. VARIATION OF "INHIBITION ACTED AT PH 8" WITH BREWING PERIOD

Months	Inactivation rate*
3	33%
6	62
9	97
12	66

* Based on the activity at pH 6.0.

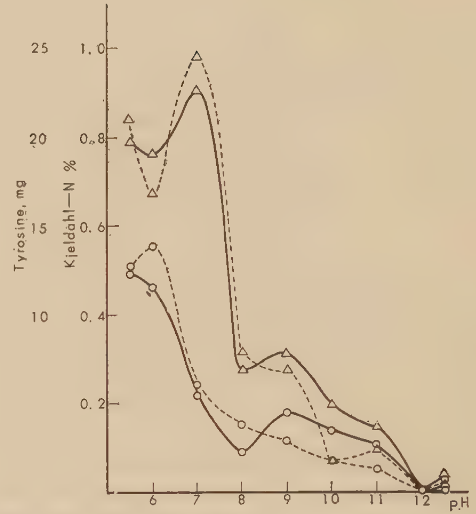

FIG. 5. The Proteinase Activity Curves of a Soy Mash showing the Inactivation at pH 10.

—○— Kjeldahl-N } 2 h enzymation
---○--- Anson's method
—△— Kjeldahl-N } 6 h enzymation
---△--- Anson's method

supernatant, proteolytic activity was reduced to less than half (Table IV).

The above mentioned facts show that the trough at pH 8.0 is probably due to an inhibitor acting specifically at pH 8 against *Aspergillus* proteinase.

The amount of this inhibitor observed in the soy mash varied widely with the brewing period (Table V).

This phenomenon was not observed on some other factory soy mashes. Also, in case of soy mash prepared with *Asp. sojae* mutant X-816[19] this trough was not observed (Fig. 2).

5. Two Peaks of *Aspergillus* Alkaline Proteinase. The proteinases of *Asp. sojae* show a high plateau of optimal pH from 6 to 11(18), but they were lowered during a long brewing period of one year in soy mash showing two eminent peaks at pH 6–7 and at pH 10 (Fig. 1, 2). At pH 10.0, the ratio of the liberation of phenolic amino acids was greater than that at pH 7.0 (Fig. 4). In a case of soy mash, inactivation of the proteinase at pH 10.0 was observed but could not be observed at pH 7.0 (Fig. 5).

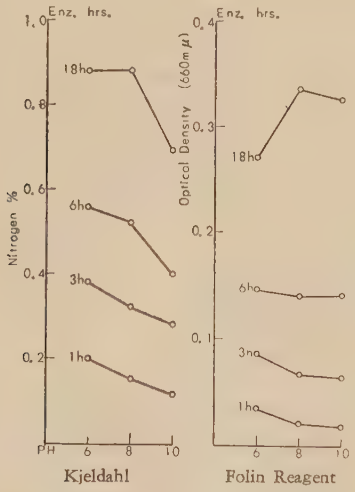

FIG. 4. Proteinase Activity Measured by Kjeldahl-N and by Folin's Reagent.

Samples are TCA 0.2 M soluble Hammersten casein hydrolyzed with a sample of soy mash.

These facts suggest that there may be two kinds of proteinases in the so-called "*Aspergillus* Alkaline Proteinase" as assumed by Crewther[15] and Wallenfels[16], one having its opt. pH at 6–7, the other at 10. Both probably differ from *Aspergillus* Neutral Proteinase[12], because of its low content in soy koji[21].

6. The Existence of *Bacilli*-spores in the Soy Mash. The foregoing experiments concerning the proteinase in the soy mash had failed to detect the action of bacterial proteinases in soy mash. However, the number of aerobic bacteria, mostly *Bacilli*, were found to be 10^5–10^8 cells per ml of soy mash when plated with the media containing less than 10% salt

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[21] K. Yamamoto, This Bulletin, **21**, 308, 313, 319 (1957).

TABLE VI. VIABLE COUNTS OF AEROBIC BACTERIA IN SOY MASH PLATED ON THE MEDIA CONTAINING VARIED SALT CONCENTRATION

Brewed months / NaCl conc. in media	2	6	12	15
0.5 %	5.0×10^{7} counts/ml	1.6×10^{6}	7.6×10^{5}	3.9×10^{7}
10.0	3.4×10^{7}	7.7×10^{5}	4.6×10^{5}	5.0×10^{7}
15.0	<100	3.1×10^{4}	1.5×10^{3}	1.5×10^{4}
18.0	<100	3.7×10^{3}	5.0×10^{2}	4.7×10^{3}
20.0	<100	<100	<100	<100
Thoma count	Rod shaped cells were difficult to observe.			

(Table VI). In spite of this, by the direct observation of soy mash under the microscope, real vegetative rod shaped cells could hardly be found, and *Pediococci*, soy sauce lactic acid bacteria[19], seemed to be predominant in the visual field.

TABLE VII. VIABLE COUNTS OF *Bacillus* SPORES IN THE SOY MASH

Soy mash	Heat shocked 80° C. 30 min.	Not heated
A	50 colonies/plate	62
B	101	102
C	19	15
D	44	35

Then, the supposition was made that most *Bacilli* might be surviving in the form of spores in soy mash. Four samples of soy mash were diluted with 6 % salted broth (in order to avoid the damage of salt tolerant bacteria), put into test tubes, heated for thirty minutes in a water bath at 80°C, and were plated out. The mean counts of five replicate plates are listed in Table VII. Since there were no significant difference in the number of colonies between the heated and unheated soy mash, it would be apparent that most of the aerobic bacilli in soy mash are in the form of spores.

DISCUSSION

It has been confirmed that the proteinases in the soy mash are mostly of *Aspergillus* proteinase origin which are produced in the stage of koji making. The proteinases were transformed, inactivated by the acidic environment and inhibitors, but still remained after one year of brewing.

Detection of the expected bacterial proteinase resulted in failure.

Bacilli, which had been confirmed to be the main bacteria in the soy sauce brewing, did not easily grow as vegetative cells in soy mash was also proved, but at least major cells of them survived in the from of spores. However in another experiment, about a half of the *Bacillus* strains isolated from soy mash could grow on the glucose supplemented nutritive agar slant of pH 7.0 containing 17 % salt. However, these strains were acid sensitive and most of them could not grow at pH 6.0. Also, from microscopic observation of the soy mash, rod shaped cells of *Bacilli* could hardly be found. It is reasonable that the environment of soy mash, which is usually 17% salt concentration and pH 4.8, or a lower pH value, is a very severe condition for the growth of aerobic bacilli. Yoshii and Nakano[22] reported that viable counts of aerobic bacteria in soy mash do not change throughout a brewing period of a year. This is in accordance with the view that most of them are in the spore form. It is considered that the aerobic bacilli in soy mash grow in the stage of koji making, but vegetative bacterial cells are killed when they are soaked in the brine having a high salt concentration, and thus, only *Bacilli*-spores survive.

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[22] H. Yoshii and M. Nakano, *J. Ferment. Technol. Japan*, **34**, 361 (1956).

It is however apparent, that the soy sauce is not solely made of the enzymes of koji mold *Aspergillus.* In the purely cultured soy koji digest, proteins contained in raw materials were dissolved into the juice as well as in the usual soy mash, but the ratio of amino acids in the juice was lower and the content of large peptides was greater than usual soy mash juice⁹⁾. It also presented a strange flavor because of the lack of lactic acid bacteria and yeast fermentation. So, it is conceivable that though small amounts they may be, the proteinases of *Bacilli* grown in the koji making stage and the intracellular proteolytic enzymes of the soy sauce lactic acid bacteria can split some of the specific peptide bonds which are resistant to *Aspergillus* proteinases, and thus bring a gain in the amino acid ratio and clearance of protein-like clouds in soy sauce. The existence and the roles of anaerobic bacteria will be discussed later.

SUMMARY

(1) Proteinase activities in the soy mash juice were measured by a modification of the Anson method, and compared with those of the purely cultured soy koji digest which contained only *Aspergillus* proteinases.

(2) The pH-activity curves, the inhibition by specific inhibitors, acid and alkali treatments showed that the proteinases in the soy mash were almost of *Aspergillus* origin. Detection of bacterial proteinases was unsuccessful.

(3) The existence of a specific inhibitor was recognized in the soy mash of *Aspergillus sojae* strain KS, which inhibits *Aspergillus* proteinase, specifically at pH 8.

(4) The two peaks of activities of *Aspergillus* Alkaline Proteinase at pH 10.0 and at pH 7.0 had a different liberation ratio of phenolic amino acids. In some cases, the inhibition of soy mash proteinase was observed at pH 10.0 but not at pH 7.0.

(5) Bacterial viable counts of soy mash juice was not changed by heat shock. The vegetative cells of *Bacilli* were difficult to observe under the microscope. It is therefore considered that most of the *Bacilli* survive in the form of spores which had been produced in the stage of koji making, and that there are very few *Bacilli* growing in the soy mash.

The author wishes to express his hearty thanks to Prof. T. Asai for his kind guidance. Thanks are also due to Prof. K. Sakaguchi, Prof. Y. Sumiki, Prof. K. Kitahara, Assist. Prof. B. Maruo, Mr. T. Yasui, Dr. T. Mogi, Dr. F. Yoshida and Mr. H. Onishi for their valuable suggestions.

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[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 6, p. 353~362, 1958]

Studies on the Activities of Bacteria in Soy Sauce Brewing

Part III. Taxonomic Studies on *Pediococcus soyae* nov. sp., the Soy Sauce Lactic Acid Bacteria

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Thirteen strains of salt tolerant specific lactic acid bacteria were isolated from soy sauce brewing mashes, and their taxonomical characteristics investigated. These strains are spherically shaped, generally assume a tetrad form, micoaerophilic, and perform the homo-type fermentation. Eleven strains produce *d*-lactic and two other strains produce *dl*+(*d*) lactic acid. These characters are unaffected even when grown in salted broth containing 15% sodium chloride. They grow well in a medium containing 18% salt, and the upper limit of the salt tolerancy is approximately between 24 to 26% sodium chloride. The salt tolerant capacity seems to be a hereditable character. The strains grow within a pH range between 5.5 to 9.0, but no growth is observed at pH 5.0. The optimal temperature was from 20°C~30°C. Many sugars are well fermented, and this ability was not diminished in 17% salted media.

From these facts, the author proposed the name *Pediococcus soyae* nov. sp. for this sort of organism.

This bacteria requires two specific nutrients: namely, a peptide-like substance (P-factor) included in the "Polypepton" (Takeda) and a dialyzable unknown substance (S-factor) included in the "Heart infusion broth", "Yeast extract" and "Beef extract" (Difco). This bacteria do not require leucovorin.

In the previous paper[1] it was reported that the *Bacillus subtilis-mesentericus* group, which had formerly been accepted as the principal soy sauce bacteria, grew only in the soy-koji-making stage, and their spores survived only in the soy mash, and that the most active growing organism in the soy mash was the salt tolerant lactic acid bacteria. This paper elucidates the taxonomical characteristics of the lactic acid bacteria and proposes the name *Pediococcus soyae* nov. sp. for this sort of organism. In this paper, two specific nutritional requirements of this bacteria will be also referred to.

Ishimaru[2] in his laborious studies reported on four varieties of soy sauce lactic acid bacteria, namely, *Pediococcus acidi lactici* var. soya α and β, *Pediococcus albus* var. soya α and β. It is assumed that they may probably be identical or at least very similar species with the author's strains, but the general taxonomical system has recently changed and moreover, two important taxonomical characteristics given in his description that their catalase activities were positive and the reduction of nitrates, were not confirmed in this study.

EXPERIMENTAL

Method of isolation. Many varieties of fresh and old soy mashes were suitably diluted, inoculated in the following media, the tube cultured in high layer and the surface was covered by the uninoculated agar. Composition of the media: Heart infusion broth (Difco) 2.5%, polypepton (Takeda) 1.0%, rice koji water (Bllg. 10°) 10.0%, dipotassium phosphate 0.5%, sodium chloride 0.5% or 7.0%, sodium thioglycollate 0.1%, agar 1.3 % and eurocidin[3] 30 γ/ml, pH 7.0. When small colonies had appeared in the agar after the incubation for about six days at 30°C, the bottom of the test tube was aseptically cracked and the colonies were hooked out with platimum needle. Purification of the organisms was carried out several times in the same manner. Each isolated organism was stab-cultured in the same media for stocking, and to estimate optimal temperature, optimal hydrogen ion concentration and salt tolerancy.

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[1] K. Sakaguchi, This Bulletin, **22**, 345 (1958).

[2] Y. Ishimaru, *J. Agr. Chem. Soc. Japan*, **9**, 859, 953, 1143 (1933).

Fermentation test. Because of the acid sensitive nature of these organisms, a mere addition of calcium carbonate in the fermentation media could not maintain its pH value high enough for full growth, so occasional addition of concentrated sodium hydroxide solution was necessary to keep the media in an appropriate pH range.

The organism was inoculated in the following medium and cultured, anaerobically from two to four months at 30°C. Medium: Heart infusion broth (Difco) 10 g, polypepton (Takeda) 4 g. glucose 40 g, dipotassium phosphate 2 g, salt 2 g, 28 g or 60 g, calcium carbonate 10 g, brom thymol blue solution 5 drops, made up to 400 ml with distilled water, pH 7.0. The medium was sterilized with a Chamberland type filtration sterilizer (Nippon Kagaku Togyo).

Fermentability of sugars. This experiment was carried out according to the method of Kitahara[4~7]. The basal medium was beef extract (Difco) 0.3 %, polypepton (Takeda) 1.0 %, dipotassium phosphate 0.5 %, salt 0.5 % or 17.0 %, brom cresol purple, pH 7.0. Five ml of the basal medium was added with 2 % sugars, autoclaved, inoculated with washed organisms, incubated anaerobically for three weeks and the acid formed titrated with N/20 sodium hydroxide. The sugars used were the products of Merck Co.[8,9], Kahlbaum Co. or Difco Lab..

Other general properties of bacteria. Methods described in the "Manual of Microbiological Methods"[9] and in the papers of Kitahara on lactic acid bateria[4~8] were taken into consideration.

RESULTS

Strains Fifteen strains of lactic acid bacteria were newly isolated from manifold samples of soy mashes collected from soy sauce factories in Noda City. Among these strains, strain *d*7 was an isolate from the mash of semi-acid-hydrolyzed soy sauce.

Salt tolerance and form of the bacteria. Amongst the 15 strains of newly isolated lactic acid bacteria, 13 strains occurred as spheres of 0.6–0.8 microns in diameter, in the tetrad form, and were supposed to be of a *Pediococcus.* sp.. The other two strains were a rod and a coccus forming short chains. The latter two strains were salt sensitive and could not grow in 7 % or more salted media. They were probably the survivors of the salt sensitive lactic acid bacteria grown in the soy koji[1,10] making stage.

The 13 salt tolerant strains grew well in 18 % salted media, and the upper limit of the salt tolerance was 24–26 % in stab-culturing. On strains *d*2 and *d*3, the growth in 24 % salted broth was also confirmed. The following studies were conducted on these 13 salt tolerant strains.

Inheritance of salt tolerance. After strains *d*2, *d*10 and *dl*2 were transplanted for 31 times successively in 0.5 % salted medium and suddenly transferred in a 22 % salted medium, normal growth was observed.

General characteristics.

Liquid culture. Under anaerobic conditions, good growth was observed in glucose supplemented nutrient broth and yeast extract broth within four days at 30°C, while growth was very poor in a synthetic medium for bioassay[11,12].

No growth on liquid surface.

The turbidity of the medium decreased after growth due to sedimentation of the organism, as white to yellowish viscous precipitate at the bottom.

Solid culture. When stabbed in glucose supplemented nutrient agar, all strains grew

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3) K. Sakaguchi, *J. Agr. Chem. Soc. Japan*, **28**, 758 (1954); *Rep. Noda Inst. Sci. Res.* **2**, (1958).

4) K. Kitahara et at., *J. Gen. Appl. Microbiol.*, **3**, 93, 102 (1957).

5) K. Kitahara, *J. Agr. Chem. Soc. Japan*, **14**, 752 (1938), **15**, 19 (1939).

6) H. Katagiri and K. Kitahara, *ibid.*, **14**, 645, 1072 (1940).

7) H. Katagiri and K. Kitahata, *Biochem. J.*, **31**, 900 (1940).

8) K. Kitahara, *J. Agr. Chem. Soc. Japan*, **16**, 697, 819 (1940).

9) Society of American bacteriologists, "Manual of Microbiological Methods," McGrow-Hill Book Co., (1957).

10) K. Sakaguchi, This Bulletin, in press. (Part IV)

11) G. Tamura et al., *J. Agr. Chem. Soc. Japan*, **26**, 464, 474 (1952).

12) E. E. Snell, *Nutrition Abstr. & Rev.*, **16**, 497 (1946-47).

TABLE I. FERMENTATION PRODUCTS FROM GLUCOSE

	Glucose consumed	Ethanol	Acetic acid	Lactic acid	Optical rotation
d 1	20.8 g	0.40 g	1.92 g	16.8 g	*d*
d 2	23.2	0.52	1.48	19.3	*d*
d 3	22.0	0.48	1.16	18.0	*d*
d 10	28.0	0.42	0.80	22.8	*d*
dl 1	27.2	0.35	0.88	24.6	*dl*+(*d*)
dl 2	27.7	0.45	1.47	21.0	*dl*+(*d*)

uniformly along the stab canal in filiform or papillate form and never revealed remarkable surface or bottom growth.

Morphology and physiology. Morphological and physiological characters were examined on the organisms cultured for four days at 30°C in heart infusion glucose broth. All strains were spheres having 0.6–0.8 μ in diameter. Most of them occurred in the tetrad form, but some were in the diploid form. Clusters were often formed, but chains were never observed. In salted media and in the soy mash the diploid form was prevailing. Non-motile. Gram stain-positive. Catalase-negative. Indole formation and V. P. test-negative. Nitrates were not reduced. Methyl red test-positive. No growth observed in litmus milk.

Gas formation. From the application of Kitahara's method[4)], no gas-formation was observed from glucose.

Growth in wort, hopped wort and beer. According to Bergey's Manual (1957)[13)], the genus *Pediococcus* is divided into two species by their optimum temperature, growth in wort, hopped wort and beer. The author's strains were acid sensitive, thus it was apparent that they could not grow in such a low pH medium as beer.

Wort, hopped wort and beer were supplied by the Azumabashi Factory of Asahi Beer Co. Ltd.. When the pH of wort and hopped wort exceeded pH 5.5, growth was observed after five days' anaerobic incubation at 30°C. No growth occurred in beer.

Fermentation formula and optical properties of produced lactic acid. Table I shows that the salt tolerant strains are to be considered to come in the category of lactic acid bacteria which conduct a homo type fermentation. The optical rotation of lactic acid produced was as follows: 11 strains, namely from strain *d* 1 to *d* 11, produced dextrorotatory lactic acid, but the other two, strain *dl* 1 and *dl* 2, produced optically inactive lactic acid along with a trace of dextrorotatory lactic acid. The optical rotation was decided by $[\alpha]_D$ and the content of crystalline water of the zinc salt.

The fact that the majority of these strains produce *d* lactic acid from glucose is a reason for differentiating these strains from *Pediococcus cerevisiae* of Pederson[13~15)].

Fermentation in salted media. Three representative strains, *d* 1, *d* 2 and *dl* 1, were inoculated in 7 % and 15 % salted media and incubated anaerobically for four months at 30°C in the same manner as in the former experiment. Table II shows that the fermentation formula and the optical characters of the acid produced are not influenced even when the fermentation occurs in highly salted media. The rate of fermentation in salted media was however, slower than that in unsalted media, and another difference observed was that strain *dl* 1 produced a larger amount of *d*-lactic acid in the salted medium.

Final hydrogen ion concentration. The strains were inoculated in 0.5 % salt and 1 % glucose supplemented heart infusion broth, and incubated anaerobically for ten days at 30°C.

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13) Bergey's Manual of Determinative Bacteriology, 7 th ed., p. 529 (1957).

14) C. S. Pederson, *Bact. Rev.*, **13**, 225 (1949).

15) R. P. Tittsler and C. S. Pederson, *Bact. Rev.*, **16**, 227 (1952).

TABLE II. FERMENTATION PRODUCTS IN SALTED MEDIA

	Salt conc.	Glucose consumed	Ethanol	Acetic acid	Lactic acid	Optical rotation
d 1	7 %	8.0 g	0.08 g	1.55 g	5.12 g	*d*
	15	11.1	0.08	2.16	7.55	*d*
d 2	7	12.0	0.08	1.48	8.91	*d*
	15	10.7	0.11	1.91	6.92	*d*
dl 1	7	25.1	0.88	1.40	19.15	*dl*+(*d*)
	15	22.6	0.12	1.94	16.20	*dl*+(*d*)

The final hydrogen ion concentration of the culture solution was measured with a Beckman pH meter (Table III).

TABLE III. FINAL HYDROGEN ION CONCENTRATION

Strain	Final pH	Strain	Final pH
d 1	5.12	*d* 8	4.90
d 2	4.72	*d* 9	4.62
d 3	4.80	*d* 10	4.70
d 4	4.82	*d* 11	4.73
d 5	4.71		
d 6	4.82	*dl* 1	4.93
d 7	4.72	*dl* 2	4.93

The acid production of this organism stopped at relatively low hydrogen ion concentrations, namely at pH 4.9–4.6. This pH value corresponds tò that of the soy mash, and provides evidence that the hydrogen ion concentrations of soy mash is retained in such a range by these organisms.

Optimal hydrogen ion concentration and halophility. Buffered and pH-controled heart infusion glucose agar was added 0.5, 6.0, 12.0, and 18 % sodium chloride. Strains *d* 3, *d* 4, *d* 6 and *dl* 1 were stabbed in the agar. Growth

TABLE IV. OPTIMAL pH AND HALOPHILITY

		d 3				*d* 4				*dl* 2				*d* 6			
pH	NaCl(%)	3	6	16	30 days	3	6	16	30 days	3	6	16	30 days	3	6	16	30 days
5.0	0.5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	6	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	12	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	18	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
5.5	0.5	−	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−
	6	−	−	+	⧺	−	−	+	⧺	+	⧺	⧺	⧺	−	−	−	⧺
	12	±	+	⧺	⧺	−	−	⧺	⧺	±	⧺	⧺	⧺	−	−	−	⧺
	18	−	−	−	−	−	−	−	−	−	−	−	⧺	−	−	−	−
6.0	0.5	−	+	⧺	⧺	−	+	⧺	⧺	+	⧺	⧺	⧺	−	−	⧺	⧺
	6	+	⧺	⧺	⧺	−	⧺	⧺	⧺	⧺	⧺	⧺	⧺	−	+	⧺	⧺
	12	−	+	⧺	⧺	−	−	⧺	⧺	+	⧺	⧺	⧺	−	−	⧺	⧺
	18	−	−	+	⧺	−	−	−	⧺	−	−	⧺	⧺	−	−	−	⧺
7.0	0.5	+	⧺	⧺	⧺	+	⧺	⧺	⧺	⧺	⧺	⧺	⧺	−	+	⧺	⧺
	6	+	⧺	⧺	⧺	+	⧺	⧺	⧺	⧺	⧺	⧺	⧺	−	+	⧺	⧺
	12	−	⧺	⧺	⧺	−	⧺	⧺	⧺	+	⧺	⧺	⧺	−	+	⧺	⧺
	18	−	+	⧺	⧺	−	−	⧺	⧺	−	+	⧺	⧺	−	−	⧺	⧺
8.0	0.5	−	⧺	⧺	⧺	+	⧺	⧺	⧺	⧺	⧺	⧺	⧺	−	⧺	⧺	⧺
	6	−	⧺	⧺	⧺	⧺	⧺	⧺	⧺	⧺	⧺	⧺	⧺	±	⧺	⧺	⧺
	12	−	+	⧺	⧺	±	+	⧺	⧺	+	+	⧺	⧺	−	+	⧺	⧺
	18	−	−	−	⧺	−	−	⧺	⧺	−	+	⧺	⧺	−	−	⧺	⧺
9.0	0.5	−	−	⧺	⧺	±	⧺	⧺	⧺	+	⧺	⧺	⧺	−	+	⧺	⧺
	6	−	+	⧺	⧺	−	⧺	⧺	⧺	⧺	⧺	⧺	⧺	−	+	⧺	⧺
	12	−	−	−	−	−	+	⧺	⧺	−	+	⧺	⧺	−	−	⧺	⧺
	18	−	−	−	−	−	−	−	−	−	−	−	⧺	−	−	−	−

is recorded on Table IV. All four strains grew only in the range of pH 5.5–9.0 while none grew at pH 5.0 even after one month's incubation at 30°C. The optimum hydrogen ion concentration was pH 7.0. Acid sensitivity of these organisms probably explain its remarkable decrease in viable counts in the soy mash at latter half year of soy sauce brewing[10]. Table IV also shows that at pH 5.5, 6.0 and 9.0, these organisms grow more rapidly in 6 % and 12 % salted media than in 0.5 % salted medium. It is conceivable that this organism is not only halotolerant, but also halophile.

Optimal temperature. All the 13 strains were stabbed in 0.5 % salted heart infusion glucose agar and incubated at 10°C, 20°C, 30°C and 40°C, respectively. Table V shows that this organism has an optimal temperature range of 20°C–30°C, namely mesophilic. At 10°C and at 40°C, no growth was observed after one month's incubation.

TABLE V. EFFECT OF TEMPERATURE

Strain	20°			30°			40°		
	3	6	10 days	3	6	10 days	3	6	10 days
d 1	±	+	+	±	+	+	−	−	−
d 2	−	+	+	+	+	+	−	−	−
d 3	±	+	+	+	+	+	−	−	−
d 4	−	+	+	+	+	+	−	−	−
d 5	−	+	+	+	+	+	−	−	−
d 6	±	+	+	+	+	+	−	−	−
d 7	±	+	+	+	+	+	−	−	−
d 8	−	+	+	+	+	+	−	−	−
d 9	−	±	+	+	+	+	−	−	−
d 10	+	+	+	+	+	+	−	−	−
d 11	+	+	+	+	+	+	−	−	−
dl 1	−	±	+	+	+	+	−	−	−
dl 2	±	+	+	+	+	+	−	−	−

Fermentable Sugars. The results are shown in Table VI.

(1) This organism as well, ferments a number of sugars under salt concentrations of 0.5 % and 17 %.

(2) Pentoses are well fermented.

(3) The organism does not ferment sucrose well, but ferments maltose. This is the specific character of *Pediococcus hennebergi*[7,8].

(4) The organism can clearly be divided into two groups by mellibiose fermentability. This coincides with raffinose fermentability. Based on trehalose fermentability, another system of grouping is also considerded possible.

(5) Two strains, *d* 6 and *dl* 1, ferment starch weakly. This was confirmed by the fermentation test.[10]

(6) The organism ferments lactose only in unsalted medium.

(7) No distinction was observed between *d*-lactic acid formers and *dl*-lactic acid formers.

(8) These organisms in many points resemble with *Pediococcus hennebergi*[7,8], but do not resemble with *Pediococcus lindneri*[7,8] and *Pediococcus cerevisiae*[13~15].

Growth in sugar-free media. Strains *d* 2, *dl* 1, *Pediococcus hennebergi*, *Lactobacillus arabinosus*, *Lactobacillus acidophilus* L-25, *Leuconostoc citrovorum* L-11, *Streptococcus faecalis* R, *Lactobacillus bulgaricus* (furnished by the Institute of Applied Microbiology, University of Tokyo) were anaerobically cultured in sugar-free liquid media and growth of the 9 lactic acid bacteria was examined (Table VII). It was found that none of these could grow in the glucose-free complete synthetic medium for the bioassay[11,12]. Supplemented with 10 % polypepton (Takada), an enzymatic hydrolysate of milk casein, two strains of soy sauce lactic acid bacteria and *Lact. acidophilus* L-25 showed growth after seven days' incubation. Further supplement of 0.3 % beef extract (Difco) supported the growth of all species except *Lact. bulgaricus*. *Lact. bulgaricus* growth was restricted to the addition of glucose.

No direct reducing sugars were detectable in the three sugar-free media by Fehling-Lehman-Schoorl's sugar assay.

Nutritional Requirements. Though all 13 strains grew, growth was very poor in the complete synthetic medium for the bioassay[11,12], and two unknown substances were required for their maximal growth (Table VIII, Figs.

TABLE VI. FERMENTABLE SUGARS IN NaCl 0.5 % AND 17 % MEDIA

Strain	*d* 1		*d* 2		*d* 3		*d* 4		*d* 5		*d* 6		*d* 7		*d* 8		*d* 9		*d* 10		*d* 11		*dl* 1		*dl* 2		*P. henn.*7,8)	*P. lind.*7,8)	*P. cere.*9,10)
NaCl (%)	0.5	17	0.5	17	0.5	17	0.5	17	0.5	17	0.5	17	0.5	17	0.5	17	0.5	17	0.5	17	0.5	17	0.5	17	0.5	17	0.5	0.5	0.5
Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	+	±	+	±	+	±	+	+	+	+	+	+	+ or −
Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	−	+	−	+	−	±	+	+	+	+	+
Rhamnose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+ or −
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	−	−	±	−	±	−	±	−	±	−	±	−	−	−	±	−	−	−	−	−	−	−	−	−	±	±	−	−	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	+	+	+	−	+
Lactose	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	+	+	−	+	−	+	−	−	−	+	+	±	−	+
Mellibiose	−	−	+	+	+	+	+	+	+	+	+	+	−	−	±	+	−	−	−	−	−	−	−	−	±	+			
Raffinose	−	−	+	+	+	+	+	+	+	+	+	+	−	−	+	±	−	−	−	−	−	−	−	−	+	+	−	−	+
Treharose	+	+	+	−	+	−	+	−	+	−	±	−	+	±	+	+	−	+	+	−	+	−	−	−	+	+			
Dextrin	+	+	+	±	+	+	+	+	+	+	+	+	+	±	+	±	+	+	+	±	+	±	+	±	±	±	−	−	−
Starch	−	−	−	−	−	−	−	−	−	−	±	+	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	−	−
Glycogen	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−			
Inulin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Mannitol	−	−	+	+	+	+	+	+	+	+	±	+	−	−	+	+	+	−	+	+	+	+	−	−	+	+	−	−	−
Glycerol	−	−	+	−	+	−	+	−	+	−	−	−	−	±	−	±	−	−	−	±	−	±	−	−	−	±	±	±	
α-Me-Glucoside	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	±	+	+	+	+	+	−	+	+	+	+	−
Arbutin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+			
Salicin	±	−	±	−	+	+	±	−	±	−	+	+	±	−	+	−	+	+	±	−	+	−	−	−	+	−	+	+	+

TABLE VII. GROWTH ON SUGAR-FREE MEDIA

Strain	Culture Days	Media Synthetic medium	Polypepton 1 %	Polypepton 1 % +Beef ex. 0.3 %	Polypeptone 1 % +Beef ex. 0.3 % +Glucose 1.0 %
Ped. soyae d 4	2	−	−	−	−
	7	−	+	+	++
Ped. soyae dl 2	2	−	−	−	−
	7	−	+	+	+
Ped. hennebergi	2	−	−	±	++
	7	−	−	+	++
Lact. arabinosus	2	−	−	±	++
	7	−	−	+	++
Lact. acidophilus L-25	2	−	−	±	+++
	7	−	+	+	+++
Leuc. citrovorum L-11	2	−	−	+	++
	7	−	−	+	++
Str. faecalis R	2	−	−	±	++
	7	−	−	+	++
Lact. bulgaricus	2	−	−	−	−
	7	−	−	−	++

TABLE VIII. NUTRITIONAL REQUIREMENTS OF *Pediococcus soyae*

Strain	Synth. Medium for Bioassay	Polypepton 1 %	Heart infusion 2.5 % Polypeptone 1 %	Beef extract 0.3 % Polypepton 1 %	Yeast extract 0.3 % Polypepton 1 %
d 1	0.17	1.03	5.2	4.84	5.25
d 2	0.26	3.25	6.18	5.81	4.21
d 3	0.65	3.21	6.95	6.05	4.40
d 4	1.10	3.35	7.42	6.53	5.52
d 5	0.43	2.71	6.33	6.02	5.62
d 6	0.60	2.89	6.30	6.12	5.67
d 7	0.81	3.25	7.02	6.01	6.12
d 8	1.37	3.72	7.55	6.71	5.68
d 9	0.40	2.86	8.21	7.07	6.69
d 10	0.08	0.76	3.56	0.21	1.57
d 11	1.09	2.59	5.18	4.60	4.27
dl 1	0.62	1.70	6.01	4.13	4.94
dl 2	0.35	2.40	6.48	6.98	6.50
Ped. henn.	0.80	12.80	16.39	16.90	16.20
Ped. lind.	5.75	11.82	14.30	12.95	13.67

After 5 days anaerolic incubation at 30°C, 5 ml of media were titrated with N/20 NaOH. This table shows ml of the titration.

1 and 2). One substance is contained in polypepton (Takeda), or in milk casein hydrolysates by *Aspergillus sojae* alkaline proteinase, or in the water extract of *Asp. sojae* mold bran, and is supposed to be a peptide. The name "P-factor" was provisionaly given to this substance. The other, is a dialyzable substance contained in heart infusion broth (Difco), beef extract (Difco), yeast extract (Difco), and in soy mash. This substance was also provisionaly named "S-factor". The following substances could not be substituted for S-factor; glutamine, asparagine, oleic acid, nyacin amide, pyridoxal, vitamin K_3, choline, inositol, nucleic acid, betaine, thymine, ornithine, orotic acid, lipoic acid, mevalonic acid,

acid, leucovorin, folic acid and vitamin B_{12}. S-factor was not included in the peptonized milk (Difco) and the autoclaved tomato juice. It was reported by Felton[16] and Jensen[17,13] that *Pediococcus cerevisiae* required leucovorin for its growth. The effects of leucovorin calcium salt (1 γ/ml) and liver fraction-L (GBI) (325 γ/ml), a leucovorin and folic acid rich-fraction, were examined for the growth stimulation of *Pediococcus soyae* strain *d*4. Fig. 3 shows that, these substances do not support growth even when the P-factor is added as polypepton (Takeda). In this respect, also, the author's strains differs from *Pediococcus cerevisiae* classified by Pederson[13~15].

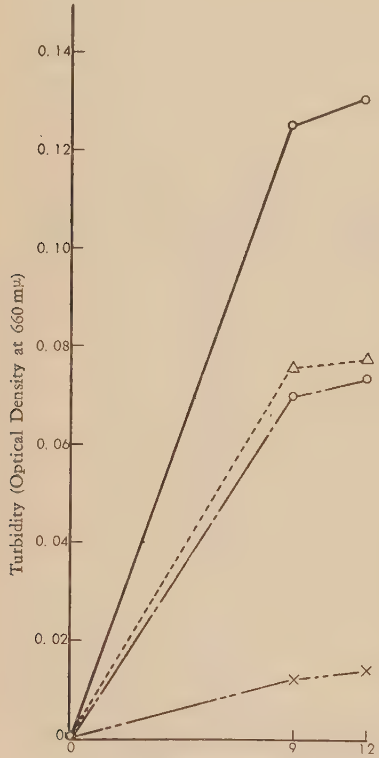

FIG. 1. Dialyzability of S-factor.

Used strain; *Pediococcus soyae dl*2

——×—— Synthetic medium for bioassay

--- △ --- Supplemented with 1 % polypepton

——○—— Supplemented with 1 % polypepton and 1 % heart infusion broth

-----○----- Supplemented with 1 % polypepton and dialyzed heart infusion broth

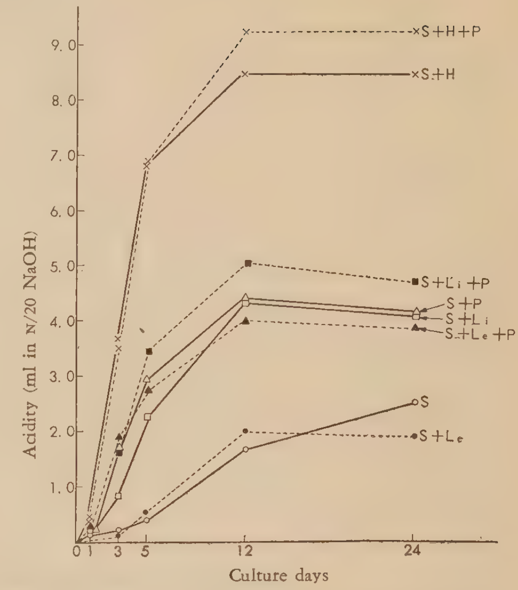

FIG. 2. Effects of leucovorin and related substances.

Used strain: *Pediococcus soyae d*4

S : Synthetic medium for bioassay

P : Polypepton 1 %

Le : Calcium salt of leucovorin 1 γ/ml

Li : Liver fraction L (GBI Co.) 325 γ/ml

H : Heart infusion broth (Difco Lab.) 25 mg/ml

Position of soy sauce lactic acid bacteria in the classification of lactic acid bacteria. The newly isolated salt tolerant tetrad strains includes 11 strains of dextrorotatory lactic acid formers, and two strains of inactive and a small amount of dextrorotatory lactic acid formers. Between the two groups, optical characteristics of lactic acid produced is the only difference hither to recognized. The fact that the majority of these strains produce *d*-lactic acid does not makes it reasonable to classify them under the genus *Pediococcus* according to Pederson[13]. However, the morphological character occurring in tetrads or in pairs but never in chains, apparently indicates that these strains should be assigned to the genus *Pediococcus*. The production of inactive lactic acid partly depends on the existence of the lacitic acid racemase[18,19],

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16) E. A. Felton and C. F. Nieven, *J. Bact.*, **65**, 482 (1953).

17) E. M. Jensen and H. W. Seeley, *J. Bact.*, **67**, 484 (1954).

and according to Kitahara's opinion[20], this is not such an important key for the classification of *Pediococci.*

Some other important characteristics of these organisms are: firstly, the high salt tolerance ranging up to 24–26 %. This distinctive character is inherited after repeated pedigree culturing in the unsalted medium.

The peculiar growth pH range of pH 5.5–9.0, acid sensitivity, requirements of two unknown nutritive substances, no leucovorin requirement and the peculiarity and importance of this organism for soy sauce brewing[21] are also a good criterion to decide these organisms to be a new species. The author, therefore, proposes the name *Pediococcus soyae* nov. sp. for these organisms.

PLATE 1.

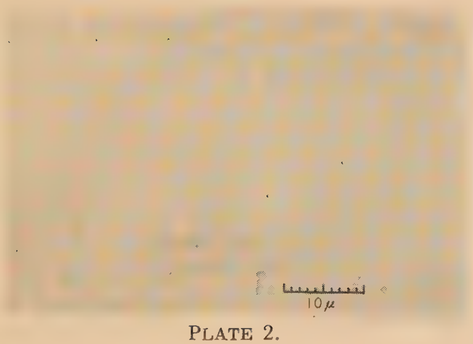

PLATE 2.

DESCRIPTION OF A NEW SPECIES

Pediococcus soyae nov. sp.

The organisms occur in a spherical form typically in tetrads, often in pairs. Never make chains. Size 0.6–0.8 microns in diameter. Old cultures form clusters. The diploid form is prevailing in salted media and in soy mash, Non motile. Gram positive.

Infusion glucose agar colonies; Pinhead, white or white yollowish, glistening, raised.

Infusion glucose agar stab; growth along stab: No surface growth observed. Filiform or papillate growth.

Infusion glucose broth: slightly turbid after four days' culture at 30°C. Afterwards, a white to yellowish white adhesive sediment is formed and the liquid medium becomes clear. Wort and beer: Moderate growth on wort and hopped wort provided its pH value are adjusted at pH 5.5 or higher.

No growth in beer.

Litmus milk: No growth.

Facultative anaerobic or microaerophilic. Catalse-negative. Nitrites not produced from nitrates. Indole-not formed. V.P. test-negative. Methyl red test-positive.

Glucose fermentation: Lactic acid and traces of ethanol and acetic acid are produced. No gas is formed. Many strains produce *d*-lactic acid, but a few strains produce *dl*+(*d*) lactic acid. Final pH value is 5.1–4.7. Acid is produced from arabinose, xylose, glucose, fructose, mannose, galactose, maltose, dextrin, α-methyl glucoside and usually arbutin and salicin in both 17 % salted and unsalted media. No acid produced from rhamnose, sucrose, glycogen and inulin. About a half of the strains ferment mellibiose, raffinose and trehalose. Some strains ferment glycerol, mannitol and starch. Lactose is fermented only in unsalted media. Optimum temperature 20°C–30°C. pH for growth: pH 5.5–9.0. Optimum pH 7.0. No growth at pH 5.0. Halophilic and halotolerant. Good growth in 18 % salted media. After a long incubation, growth in 24 %–26 % salted media. This characteristic is inheritable.

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18) H. Katagiri and K. Kitahara, *J. Agr. Chem. Soc. Japan*, **12**. 844 (1936), *Biochem. J.*, **31**, 909 (1937).

19) K. Kitahara et al, *J. Agr. Chem. Soc. Japan*, **26**, 162 (1952), **27**, 306 (1953).

20) K. Kitahara, unpublished.

21) K. Sakaguchi, This Bulletin, in press. (Part V)

Besides the ingredients of synthetic medium for bioassay, unknown growth factors of a peptide nature and a second unidentified subtance are required.

Sources: Isolated from the soy mash and and from soy-koji.

This new species is the prevailing bacteria in soy sauce brewing especially, in an earlier stage, produces lactic acid and presents good flavor.

Habitat: Soy sauce mash and soy-koji.

Acknowledgements. The author wishes to express his hearty thanks to Prof. T. Asai and Prof. K. Kitahara, University of Tokyo, for their kind guidances throughout this work. Thanks are also due to Dr. K. Sakaguchi, Prof. Y. Sumiki, Dr. M. Mogi and members of the Noda Institute for Scientific Research for their valuable suggestions. Many precious vitamins were generously supplied by Dr. G. Tamura and Mr. J. Kirimura. The author is greatly indebted to Mr. T. Kuramochi for his able technical assistance.

The author acknowledges that Mr. K. Yamazato has independently isolated almost identical, or very similar strains from the soy sauce factory in the Choshi District, Chiba Pref.

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[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 6, p. 363~368, 1958]

Biochemical Studies on Bakers' Yeast

Part I. Synchronization by Limited Feeding*

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In order to establish a synchronized state of bakers' yeast cells, environmental conditions suitable for budding and cell division were examined. It was observed that budding did not occur below a certain level of feed rate in aerated flow culture, and, this level somewhat increased at a higher temperature. In the low feed rate, separation of daughter cells from the mother cells was not significantly influenced by temperature with aeration, while it proceeded more rapidly at a higher temperature with shaking. The conclusion is that the separation of daughter cells is influenced not only by physiological conditions but also by mechanical conditions. At 35°C, however, the secondary budding took place after budded cells had divided, even at the level of feed rate, under which the budding of rest-cells could not have occurred. A preliminary treatment for synchronization was then devised, and it was concluded that the present treatment for synchronization is due to a process of slight growth, not being due to starvation.

Synchronization of microbial cells by physiological means may be differentiated into two main procedures; the one is based on the the regulation of physical conditions of cultural environment, and the other on the limitation of nutritional factors. For the former, a repeated shift of temperature is usually employed and, regarding this means, plenty of examples are to be found in the literature[1,2]. Whilst, for the latter, there are merely few examples which are performed by limitation of either nitrogen source[3,4], carbon source[5], or trace materials[6].

In regard to the synchronous culture of yeast, Ogur et al.[7] stated variations in the deoxypentose nucleic acid contents during cell growth, but they did not apply any special means for the synchronization of cells. Campbell carried out a synchronization by limitation of carbon source[5] and proceeded to report further studies on the effect of sugar concentration and pH etc.[8].

On the other hand, it is generally accepted that commercial bakers' yeast is necessary to be completely composed of resting cells without buds and, these needs are now almost satisfactorily met. Thence, the present authors after studying environmental conditions for budding and cell division, succeeded in the synchronization of bakers' yeast cells, being able to make these cells uniformally, in a state "before budding" with a limitation of medium supply.

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* This work was presented at the Annual Meeting of the Agric. Chem. Soc. of Japan, partly at the meeting held in Tokyo, on Apr. 9, 1957, and mainly at the meeting held in Kyoto, on May 3, 1958.

1) T. Yanagita, *Kōso Kenkyuhō* (*Methods in Enzymology*) Vol. III, p. 697 (1957), Tokyo, Japan.

2) A. Campbell, *Bacteriol. Revs.*, **21**, 263 (1957).

3) C. A. Beam, R. K. Mortimer, R. G. Wolfe and C. A. Tobias, *Arch. Biochem. Biophys.*, **49**, 110 (1954).

4) T. Yanagita, *Bacteriol. Proc.*, **1954**, 29.

5) A. Campbell, *Bacteriol. Proc.*, **1956**, 40.

6) H. D. Barner and S. S. Cohen, *J. Bacteriol.*, **72**, 115 (1956).

7) M. Ogur, S. Minkler and D. O. McClary, *J. Bacteriol.*, **66**, 642 (1953).

8) A. Campbell, *J. Bacteriol.*, **74**, 559 (1957).

METHODS

Strain. Yeast strain No. 23 (*Saccharomyces cerevisiae*), which is used for the production of commercial yeast at the Oriental Yeast Mfg. Co. Ltd., was supplied to samples in all experiments. Seed yeast of the factory was usually used after washing with distilled water. In some cases, it was preincubated in a synthetic medium from stock culture, and harvested after forty hours' shaking.

Cultural conditions. (1) *Flow culture:* Ten g of seed yeast was suspended in distilled water, placed in a cylindrical jar-fermenter which was placed in a thermostat, and aerated vigorously from a disk sparger made of stainless steel with about two hundred small holes (0.8 mm in diameter) on the surface of the disk (65 mm in diameter). The aeration rate was usually 30 l per minute, and pH of the medium was maintained in the range from 4.6 to 5.0; pH adjustment was accomplished by the addition of 4 N sulfuric acid or caustic soda. An aliquot-volume of the medium was placed in haematocrit every hour (fifteen minutes before feeding), being centrifuged for five minutes at 3000 r.p.m., and the weight of yeast was determined from the packed volume of the cells obtained. The feed rate for every hour was decided by the weight of yeast estimated as above. The synthetic medium of the following composition was added intermittently every thirty minutes according to the feed rate. The feed rate on the other hand, was assumed as the supplying rate of sugar, and the figures show the amount of sugar required for 1 g of moist yeast per hour.

The flow culture was also performed with shaking in a 500-ml shaking flask, just as in the case of aerated culture.

(2) *Batch culture:* In a 500-ml shaking flask, 5 ml of the synthetic medium of the following composition was placed, filled up to 100 ml with a buffer solution (the citrate buffer: final concentration ratio being 0.025 M), and inoculated with 100 mg of seed yeast. Cultivation was carried out on a reciprocating shaker at 30°. The shaker was set for 110 oscillations per minute with an amplitude of 5 cm.

Composition of medium. The composition of the basal medium was as follows: glucose 200 g, urea 17.2 g N/glucose=0.035), $NaH_2PO_4 \cdot 2H_2O$ 7 g (P/glucose=0.007), $MgSO_4 \cdot 7H_2O$ 3.8 g, KCl 2.2 g, biotin 400 μg, calcium pantothenate 10 mg, pyridoxine 20 mg, thiamine 80 mg, inositol 200 mg, $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ 3 mg, $ZnSO_4 \cdot 7H_2O$ 8 mg, $CuSO_4 \cdot 5H_2O$ 500 μg, dissolved in distilled water and filled up to 1 l. The pH of the medium was adjusted to 5.0 with 4 N sulfuric acid.

Counting of cell numbers. The cultured medium was diluted with sterilized distilled water to about an order of 10^2 cells per ml, and 1 ml of this diluted medium was placed in a petri-dish. Ten ml of the synthetic medium (containing 1 % glucose and 1.5 % of agar-agar) was then added and it was incubated for forty-eight hours at 30°.

The number of viable cells was calculated from the number of colonies appearing. At times, direct counting of total cells was made by means of Thoma's haemacytometer. The variation coefficients of vial counting were 5-9 %.

Bud ratio measurement. Direct microscopic counting of about 350 cells including buds was made in Thoma's haemacytometer, and the bud ratio was expressed as the percentage of budded cells to total cells. The variation coefficient of this procedure was 2-4 %.

RESULTS AND DISCUSSION

Rate of synchronization of commercial bakers' yeast. Ogur et al.[7] observed that when commercial bakers' yeast cells are placed in a fresh medium, almost all of the cells initiate budding at the same time about one hour

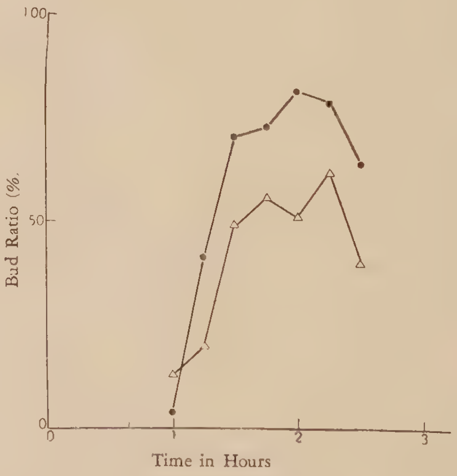

FIG. 1. Course of Budding of Treated and Untreated Cells.

Batch culture is as described in text.

—△——△—; By commercial bakers' yeast cells without any treatment.

—●——●—; By the same cells, incubated previously at a feed rate of 0.04 (30°, 5 hrs., shaking).

TABLE I. INFLUENCE OF FEED RATES AND TEMPERATURES ON BUDDING

Temp.	25°C				30°C				35°C			
Feed rate	0.04	0.08	0.12	0.16	0.04	0.08	0.12	0.16	0.04	0.08	0.12	0.16
Time hrs.												
2.0	–	–	–	–	–	–	–	±	–	–	–	+
	–	–	±	±	–	–	±	+	–	–	±	+
3.0	–	±	+	+	–	±	+	⧺	–	–	+	⧺
	–	±	+	+	–	+	⧺		–	–		
4.0	–	+	+	+	–	⧺			–	–		
	–	⧺	⧺	⧺	–				–	–		
5.0	–				–				–	–		
	–				–							
6.0	–				–							

Flow cultures under aeration, as is indicated in text. Bud ratio was examined every thirty minutes. Following notations signify bud ratio: –; new buds were not observed, ±; less than 10 %, +; 10 to 30 %, ⧺; 30 to 60 %.

later, because of its "well dissimilated state", while this was not the case in Japanese commercial bakers' yeast provided any special treatment was not applied. In the present case, the yeast displayed a sharp synchronous budding after such treatment which will be depicted in the succeeding section (Fig. 1).

Conditions for budding and "half synchronization". A feeding process is generally employed for the propagation of bakers' yeast, and one of the most serious factors controlling the growth rate is the rate of sugar supply. According to Maxon and Johnson[9], the optimum rate of sugar supply for maximum growth is 3.5 mM of glucose per 1 g of dried yeast per hour, which corresponds to 0.18 g glucose per 1 g of wet yeast containing 28 % of dry matter. When bakers' yeast cells were cultivated under various sugar supplies or feed rates, a distinctive relation between the feed rates and the appearance of budded cells was then observed. A typical example of these relations is shown in Table I. At 30°, budded cells scarcely appeared at a feed rate of 0.04 and the cells after this treatment displayed synchronous budding and cell division when batch culure inoculated with those cells was newly carried out in a fresh medium (Figs. 1, 2). In this case, a stepwise increase of cell number took place and the decrease of the bud ratio which coincided with the increase

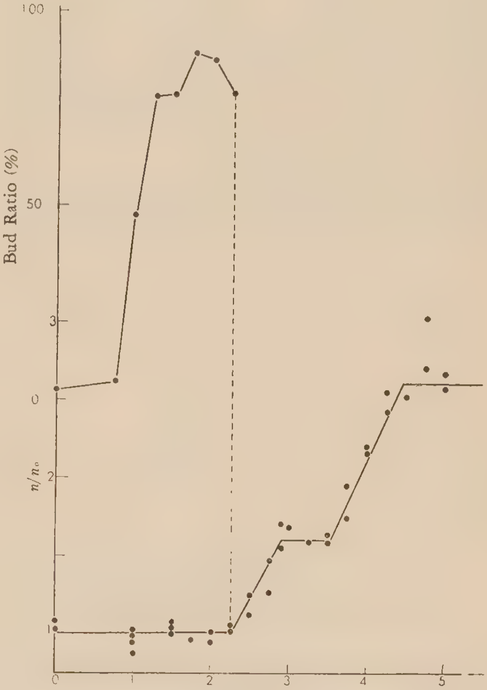

FIG. 2. Course of Multiplication of Synchronized Cells.

Batch culture is as described in text. The ratio, n/n_0, was plotted, where n indicates the number of cells at a given time and n_0 indicates the average number of cells initially existing. n_0 was $1.8 \cdot 10^8$ per ml. The upper curve indicates the bud ratio in the same culture.

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[9] W. D. Maxon and M. J. Johnson, *Ind. Eng. Chem.*, **45**, 2554 (1953).

of cell number, occurred after the bud ratio reached its maximum value (Fig. 2). Then, a synchronous state of bakers' yeast cells could well be established by four to five hours' incubation with a feed rate of 0.04 under shaking or aeration at 30°. This was only the case when commercial bakers' yeast was used as a starting material, because it ordinarily contains budded cells in a little amount or to an extent below 5 %. This process was designated as "half synchronization".

The relation between feed rates and budding was greatly influenced by temperature. At 35°, the budded cell did not appear at a feed rate of 0.08, as is shown in Table I. An incubation at 35° with a feed rate of 0.08 seemed to be more suitable for the "half synchronization" than that at 30° with a feed rate of 0.04. The time required to display budding was not affected by any of the synchronization treatments.

Conditions for cell division and "total synchronization". The step of separation of daughter cells from mother cells, i.e., the later process of the cell growth within one generation was investigated under various conditions of incubation. Several conditions, which could not produce any buds so far tested as seen from Table I, was especially adopted. It was found that the decrease of the bud ratio corresponded to the course of cell division under these conditions.

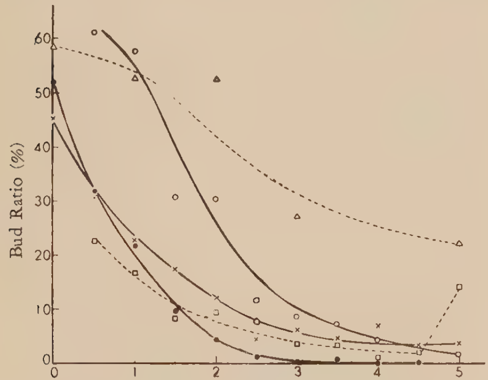

FIG. 3. Course of Cell Division under Various Conditions.

Well-grown budded cells (bud ratio in the cell population was 50~60 %) were incubated under various conditions, and the course of decrease of the bud ratio in the population was observed. Conditions which could not produce new buds from rest-cells were adopted, where the decrease in bud ratio corresponded to the course of cell division.

	Temp.	Feed rate	O_2 supply
—●——●—	35°C	0.04	aeration
—×——×—	30	〃	〃
--○--○--	33.5	〃	shaking
--△----△--	30	〃	〃
--□----□--	35	0.08	aeration

Growth and separation of daughter cells took place slowly by incubation at a feed rate of 0.04 at 30°, whereas these conditions entirely depleted the budding process of rest-cells. However, at 35° both steps proceeded somewhat rapidly as illustrated in Fig. 3. The effect of temperature on the propagation steps was observed more distinctly in the shaking culture than in aerated culture. The results obtained indicated that the separation of daughter cells may be considerably affected by mechanical conditions besides physiological conditions. In spite of no marked differences in the division rate under aeration, there always remained small amounts of budded cells after several hours' treatment at 30°, and the rate of synchronization obtained thereafter was inferior to that of the cells after treatment at 35°. In the case of 35° and a feed rate of 0.08, under which the appearance of buds from the rest-cells was not detectable, as seen

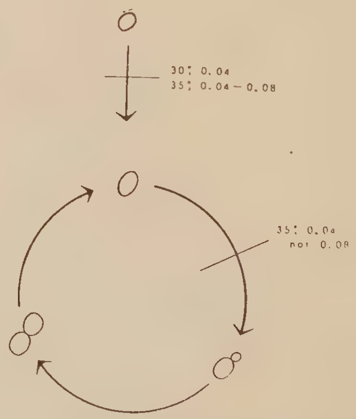

FIG. 4. Possible Scheme of Relations between Cultural Conditions and Division Cycle.

from Table I, a small amount of new buds was observed as incubation further proceeded. This may indicate that there may be relatively significant differences in the physiological properties between the rest-cells and those which have once undergone budding. This fact will also be inferred from the fact that the so-called induction or lag phase for budding was not discerned after it once took place (Fig. 2). A possible scheme on the relations between the cultural conditions and the division cycle of yeast is given in Fig. 4.

Cells in every stage of growth were able to be uniformally arranged in a stage of "before-budding" after four to five hours' treatment at a feed rate of 0.04 at 35°. Such cells display the same representative type of synchronous culture as is shown in Fig. 2, if they are newly placed in a fresh medium. This procedure is called "total synchronization".

Consideration on factors controlling synchronization. One of the authors previously observed[10] that budded cells disappear three to four hours later, if the feed rate is lowered and nitrogen supply stopped at the end-period of feeding culture. In that case, in spite of the interruption of nitrogen supply, new budded cells again appear if sugar supply is kept in optimum. There are also other examples of synchronization based on nitrogen starvation[3,4]. However, from the following facts, it might be reasonably mentioned that the present procedure of synchronization is based on the limitation of sugar supply.

1) When excess sugar was supplied and there was no nitrogen, the rest-cells initiated budding to a certain extent, utilizing their intracellular nitrogen, as pointed out by Terui et al.[11] (Table II, Exp. 1).

2) The rate of separation of daughter cells was very slow without a supply of nitrogen or phosphorus, even though excess sugar existed. At the feed rate of 0.04, the separation rate resulted to be slower with limited nitrogen than in the case with a well balanced supply of nitrogen (Table II, Exp. 2, 3).

3) "Half synchronization" was unsuccessful when either nitrogen or phosphorus was

TABLE II. INFLUENCE OF NUTRITIONAL CONDITIONS ON BUDDING AND SEPARATION OF DAUGHTER CELLS

Exp. No.	1	2		3			
Type of culture	Batch	Batch		Flow			
Feed rate	–	–		0.04			
Nitrogen	–	–	⧺	+	+	±	±
Phosphorus	⧺	⧺	–	+	±	+	±
Hrs from start							
0	4.9	39.6	39.0	57.8	58.5	58.5	58.5
2	41.9	15.0	60.7	27.4	38.1	–	44.6
4		5.8	29.4	19.7	29.9	25.5	40.4
6		3.3	15.0				

Shaking culture conducted at 30°. Quantities of existing nitrogen (supplied as urea) and phosphorus (as $NaH_2PO_4 \cdot 2H_2O$) are indicated as gram per gram of sugar supplied, and above notations are adopted here.

		Nitrogen	Phosphorus
Excess in quantities	⧺	0.12	0.08
Adequate 〃 〃	+	0.04	0.007
Slight 〃 〃	±	0.005	0.001
Absent	–	0	0

The figures in the table indicate bud ratio in per cent.

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[10] M. Hayashibe and K. Matsumoto, *J. Agric. Chem. Soc. Japan*, **32**, 594 (1958).

[11] G. Terui and H. Okada, *J. Ferm. Tech. Japan*, **29**, 435 (1951).

TABLE III. INFLUENCE OF PRIOR TREATMENTS ON BUDDING SYNCHRONIZATION

Nitrogen	+	+	±	±		−	+
Phosphorus	+	±	+	±		+	+
Time nin.							
0*	48	22	22	−	7**	20***	8****
15	71	54	40	51	30	40	75
30	82	59	58	53	60	60	80

Flow culture by shaking with a feed rate of 0.04. Temp. 30°C. Designations and figures same as those in Table II.

* Zero minute indicates time when new buds were observed for the first time.

** Untreated cells.

*** Starvation for nitrogen.

**** An example of typical budding synchronization.

TABLE IV. VARIATION OF CHEMICAL COMPOSITIONS DURING SYNCHRONIZATION TREATMENT

	Dry wt. (mg)	Total N (mg)	Amino N (mg)	Total P (mg)	RNA (mg)	DNA (mg)
Untreated cells	133	9.93	0.66	1.86	4.77	0.53
	140	11.02	0.70	2.04	5.09	0.61
Synchronized cells	161	12.60	0.99	2.12	5.50	0.45
	164	12.92	0.97	2.13	5.79	0.52

	Total carbohydrate (mg)	TCA-sol. carbohydrate (mg)	Glycogen (mg)
Untreated cells	42.4	13.3	6.4
	44.8	11.9	6.4
Synchronized cells	48.5	18.1	8.1
	51.8	17.9	9.8

After synchronization treatment (5 hours' incubation with shaking, at a feed rate of 0.04 and 30°C), the cells were harvested and washed with cold water. The chemical compositions of the untreated cells, and those of the synchronized cells corresponding to the untreated cell were estimated at the same time as follows. Total nitrogen and phosphorus: Determined by micro-Kjeldahl's and Fiske-Subbarow's[12] methods, respectively, after digestion with sulfuric acid. Amino acid pool: Determined by Pope-Stevens' Cu-method[13] for the extracts obtained by 7 % trichloracetic acid at 70°, 10 minutes. RNA and DNA: by extinction at 260 mμ, for the fractions obtained by Schmidt-Thannhauser[14]. Carbohydrates: Determined by anthronemethod[15], for the fractions obtained by Trevelyan-Harrison[16]. Duplicate analysis were carried out with different incubations.

omitted or limited at the feed rate of 0.04 (Table III).

4) A chemical composition of the synchronized cells indicated that "half synchronization" was not a process of starvation but a growth process (Table IV).

It was concluded that a limited sugar supply of well-balanced medium is requisite for the present synchronization, and that slight growth which does not accompany budding may make cells uniform in a stage of "before-budding".

Acknowledgement. The authors wish to express their thanks to Mr. T. Shōda, the director of Oriental Yeast Manufacturing Company, for the opportunity to publish this report. Grateful thanks are also due to Mr. K. Miyakawa, chief of this department, Dr. S. Nishimura, former chief of this department, Prof. S. Akabori and Prof. K. Okunuki, Osaka University, and Prof. T. Uemura, Tohoku University, for their constant encouragement and guidance. They also wish to express their sincere gratitude to Mr. T. Matsuo and Miss A. Tagaya for their technical assistance.

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12) C.H. Fiske and Y. Subbarow, *J Biol. Chem.*, **66**, 375 (1925).
13) C.G. Pope and M.F. Stevens, *Biochem. J.*, **33**, 1070 (1939).
14) G. Schmidt and S. G. Thannhauser, *J. Biol. Chem.*, **161**, 83 (1945).
15) R. Dreywood, *Ind. Eng. Chem.* (*Anal. ed.*), **18**, 499 (1946).
16) W. E. Trevelyan and J. S. Harrison, *Biochem. J.*, **50**, 298 (1952).

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 6, p. 369~372, 1958]

Carbohydrate Metabolism by *Acetobacter* Species

Part I. Oxidative Activity for Various Carbohydrates

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Many strains of *Acetobacter* were isolated and collected from various habitats, twenty of them being selected for the present study; oxidizabilities of the resting cell for various substrates were measured by the manometric method. Thus, it was observed that *Acetobacter* species are distinguishable as two groups by their oxidizabilities for carbohydrates and alcohols.

Bacteria belonging to *Acetobacter* genus are one of the useful microorganisms. They have been used in the production of vinegar since olden times because they can oxidize ethanol to acetic acid[1,2], and moreover, have been applied in the gluconic acid- or sorbose-industries since it had been pointed out that they have strong oxidative activity for some sugars. Up to the present, their microbiological natures have been reported by many investigators. Recently, however, it has been observed that the pressure of oxygen has strong influences on the metabolisms of most micro-organisms except anaerobes; therefore, for example, the incubating technique is changing from stationary to submurging in the industry concerned. On the aerobic bacteria, the oxidative fermentations by *Pseudomonas*[3,4], *Aerobacter*[5,6], *Serratia*[7,8], and so on, under a high oxygen pressure have been recognized, which is breaking new ground in the study on aerobial metabolic function of sugars and in the application of aerobic bacteria to the above industries. Especially, *Acetobacter* species are the most fundamental oxidative bacteria and sugar metabolism by them is very specific, in comparison with that by other aerobes, and they have a rich variety in regard of the oxidative systems. Therefore, from a new point of view of comparative biochemisty, the authors have attempted to investigate, in the mass, the metabolic patterns of sugar oxidation by many *Acetobacter* species.

EXPERIMENTAL AND RESULTS

First of all, to collect as many strains as possible, strains were isolated from many flowers and fruits produced in each season, such as acidified Japanese saké, beer, and vinegar etc. Walt (Bllg. 5~6°) with calcium carbonate and further with ethanol were used as isolating media. The authors then isolated sixteen strains from Japanese saké, vinegar, and other fermentative products, and fifty-seven strains from flowers and fruits. Of these strains, twenty species were selected and identified. Numbers and the habitats of isolated analogous strains are represented in Table I.

There were three new species and one varient, named by the authors respectively: *Acetobacter rubiginosus*, *Acetobacter aurantium*, *Acetobacter albidus*, and *Acetobacter suboxydans α*.

***Acetobacter rubiginosus* nov. sp.** Isolated from *Eritotrya japonica* Lindl.; Rods, 0.6 to 0.8 by 0.8 to 1.5 micron, generally occurring singly or in pairs, rarely long cell. Non motile. In wort, strong uniform turbid with thin ring formation. Orange brown pigment produced. Wort agar colonies: surface smooth,

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[1] F. T. Kützing, *J. f. pr. chem.*, **11**, 385 (1837).
[2] L. Pasteur, *Compt. rend.*, **48**, 337 (1859).
[3] V. V. Pervozvanski, *Microbiologiya, USSR*, **8**, 149 (1939).
[4] L. B. Lockwood, *J. Bacteriol.*, **42**, 51 (1941).
[5] E. Masuo, *Anual Rep. Shionogi Res. Lab.*, **3**, 291 (1953).
[6] H. Katagiri and S. Tochikura, This Bulletin, **21**, 210, 215 (1957).
[7] Y. Ikeda, *J. Agr. Chem. Soc. Japan*, **24**, 56 (1950).
[8] T. Asai, K. Aida and Z. Sugisaki, *ibid.*, **27**, 129 (1953).

TABLE I.

Species No.	Name	Numbers of isolated analogue F.P.*	F.F.*
1	*Acetobacter ascendens* HENNEBERG	1	
2	*A. aceti* BEIJERINCK	3	
3	*A. pasteurianum* HANSEN	4	
4	*A. kützingianum* HANSEN	3	
5	*A. rancens* BEIJERINCK	5	
6	*A. melanogenum* BEIJERINCK		4
7	*A. rubiginosus* nov. sp.		5
8	*A. aurantium* nov. sp.		4
9	*A. acetosum* HENNEBERG		3
10	*A. dioxyacetonicus* ASAI		3
11	*A. orleanens* HENNEBERG		2
12	*A. cerinus* ASAI		8
13	*A. gluconicum* HERMANN		4
14	*A. xylinum* BROWN		3
15	*A. industrium* HENNEBERG		5
16	*A. oxydans* HENNEBERG		4
17	*A. roseus* ASAI		5
18	*A. albidus* nov. sp.		2
19	*A. suboxydans* KLUYVER		3
20	*A. suboxydans* α nov. var.		2

* Habitat is shown F. P. is fermentative products, F. F. is flowers and fruits.

flat, entire, glistening, color of colonies is grayish white later becoming reddish brown. No growth observed in Henneberg's, Janke's, Hyduck's, and Beijerinck's media. Does not utilize ammonium salt. Oxidizes alcohol to acetic acid but does not oxidize acetic acid. Gluconic, 2-ketogluconic, and 2, 5-diketogluconic acids produced from glucose. Oxidizes mannitol to fructose; sorbitol to sorbose; glycerol to dihydroxyacetone. Produces acid from maltose strongly. Differs from known analogous species as follows.

	Gelain liquefaction	Acid production from maltose
A. melanogenum Beijerinck[9]	−	+
Gluconoacetobacter liquefaciens Asai[10]	+	−
A. rubiginosus nov. sp.	+	+

Distinctive character: The formation of dark brown to black pigment in media containing glucose with calcium carbonate. Gelatin liquefies. A large amount of acid is produced from maltose.

***Acetobacter aurantium* nov. sp.** Isolated from *Lilium auratum* Lindl.; Rods occurring singly or in pairs, 0.7 to 0.8 by 1.0 to 1.2 micron. Wort agar colonies: surface smooth, flat, entire, glistening. Yellowish brown pigments produced. In wort, moderate, turbid with orange brown pellicle formation, orange brown pigment produced. Grow in Henneberg's and Hayduck's media. Utilize ammonium salt. Liquefies gelatin. Oxidizes alcohol to acetic acid, but not acetic acid. Oxidizes glucose into gluconic acid but not into 5-ketogluconic acid, produces a large amount of unknown reducing acid. Especially, oxidizes pentoses; xylose, arabinose, etc. well. Distinctive characters; produces brown pigment in media containing glucose with calcium carbonate. Oxidized glucose into gluconic and good deal of unknown reducing acid. Oxidative ability of pentoses is very strong. Utilizes ammonium salt.

***Acetobacter albidus* nov. sp.** Isolated from *Dahlia coccinca* Cav.; Rods, occurring singly or in pairs, rarely long cell, 0.6 to 0.8 by 1.0 to 1.5 micron. Wort agar colonies: milky white, smooth, flat, very glistening. In wort forms a thin ring. No growth in artificial media and then does not utilize ammonium salt. Opt temp. for growth at 30 to 35°C. Does not oxidize acetic acid but strongly oxidizes sugars. Mainly produces 5-ketogluconic acid from glucose, fructose from mannitol, sorbose from sorbitol, dihydroxyacetone from glycerol, kojic acid from sorbose, and a great deal of acid from maltose. Distinctive character: color of colonies is milky white, and a great deal of acid produced from maltose.

***Acetobacter suboxydans* α nov. var.** Isolated from *Fragaria chiloensis* Duch.; Rods occurring singly or in pairs, 0.7 to 0.8 by 1.5 to 2.0 micron. Non motile. Scant turbid with thick rind formation in wort. Wort agar colonies: grayish white, smooth, flat, glistening. Does not utilize ammonium salt. Opt. temp. 30°C. Strongly oxidizes sugars. Produces 5-ketogluconic acid from glucose, fructose from mannitol, sorbose from sorbitol, dihydroxyacetone from glycerol, especially, Ca-5-ketogluconate crystals are formed on the surface of agar slants containing glucose and calcium carbonate. Resembles *A. suboxydans*, but differs from this species as follows. In stationary culture it does not oxidize amyl alcohol and also ethanol which are oxidized by *A. suboxydans*[11,12]. Produces 5-ketogluconic acid from glucose quantitatively. Distinctive character:

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9) M. W. Beijerinck, *Zbl. Bakt.*, II, **4**, 209 (1898).
10) T. Asai, *J. Agr. Chem. Soc. Japan*, **11**, 610 (1935)
11) A. J. Kluyver, *Zbl. Bakt.*, II, **68**, 404 (1926).
12) A. J. Kluyver, *Die Deutsch Essig Ind.*, **29**, 175 (1925).

5-ketogluconic acid produced from glucose quantitatively. Does not oxidize amyl alcohol and oxidative activity of ethanol is very slight.

Since the oxidative activity of *Acetobacter* species for various carbohydrates probably forms an important basis for the classification of this genus, the authors have undertaken experiments on about twenty species mentioned above to measure the oxygen consumption for many carbohydrates by their cell suspension using Warburg's manometer, and to compare the incubating results mentioned below.

Selection of the incubating media: Experimental media were selected first of all, because it was observed that some media had changed the oxidizing activity of bacteria in the incubation extending over successive generations. The malt or koji extract with calcium carbonate was found best to maintain bacteria for a long period in a stable state, so it was used in the following experiments.

Preparation of cell suspension: Each species, which was trans-incubated on the slant-media containing malt extract (Bllg. 6°) with 1% calcium carbonate every month, was pre-incubated on the same slants at 30°C for two days. The cells were mildly gathered up by loop and suspended homogeneously in distilled water. After agar impurities were removed through an absorbent cotton, cells were collected centrifugally and washed with water twice, followed by suspension in fertilized water.

Contents in vessels:

Cell suspension	1.0 ml
M/15 Phosphate buffer (pH 6.5)	1.5 ml
M/10 Substrate solution	0.3 ml

(20% Potassium hydroxide, 0.2 ml in centre well)

Acids contained in the substrates were used as the sodium salt. The experimental temperature was 30°C.

TABLE II. OXYGEN CONSUMPTION (Q_{O_2}) FOR VARIOUS SUBSTRATE BY *Acetobacter* SPECIES

Species No.	Group 1				Group 2											
	1	2	3,4	5	6	7	8	9,10	11	12	13,14	15	16,17	18	19	20
maltose	6	6	5	4	20	130	30	10	10	13	3	30	5	140	20	14
sucrose	7	7	7	6	130	130	220	60	80	180	100	32	140	40	170	200
glucose	50	130	100	120	520	540	480	550	530	520	540	548	560	450	540	540
gluconic acid	3	10	2	4	340	250	130	360	360	320	380	452	360	200	240	150
galactose	8	2	6	8	50	30	250	30	40	78	60	57	60	30	27	30
mannose	6	3	3	3	30	20	300	20	20	25	20	30	30	20	35	35
fructose	3	7	5	9	90	170	270	9	20	340	370	485	450	370	470	470
sorbose	6	2	4	5	10	20	10	8	17	40	50	60	30	250	410	450
L-arabinose	6	3	6	9	40	7	320	2	20	60	40	50	40	6	25	30
D-xylose	2	7	6	6	50	15	330	6	30	70	50	60	50	11	25	20
sorbitol	7	8	8	8	120	100	60	60	60	330	360	450	400	350	530	540
mannitol	6	7	5	7	100	200	40	50	70	380	480	504	420	360	740	550
glycerol	30	60	50	40	200	230	70	100	60	400	420	470	450	300	530	540
succinic acid	150	250	170	200	10	7	7	7	7	10	6	10	7	4	5	4
fumaric acid	100	200	80	130	8	6	7	5	7	9	5	8	6	7	7	6
malic acid	80	120	60	100	7	4	6	5	6	6	5	7	5	5	6	6
citric acid	2	2	2	2	3	2	2	2	2	11	2	7	2	3	2	2
pyruvic acid	100	120	80	190	210	80	100	60	7	10	4	6	4	5	7	6
acetic acid	80	370	90	100	30	20	10	20	6	8	3	8	10	5	3	4
lactic acid	250	290	260	450	370	50	40	120	50	20	7	40	20	20	25	30
ethyl alcohol	530	560	550	540	450	460	300	500	500	460	450	420	460	430	460	50
propyl alcohol	500	540	520	520	410	420	300	480	470	450	430	450	430	400	450	50
iso-propyl alcohol	20	40	20	15	80	60	30	40	70	130	140	120	100	100	100	100
buthyl alcohol	450	500	450	480	420	410	302	460	450	430	420	300	400	420	440	50
amyl alcohol	260	340	60	40	370	360	170	350	400	400	400	207	350	400	400	3

DISCUSSION

It has been observed that the metabolic activity of microorganisms is influenced by their growth environment. As shown in Table II, it was recognized that even in one genus of *Acetobacter*, the habitat strongly affected their metabolic acitivity. Namely, as reported by Asai[13], the species isolated from the substances containing ethanol or acetic acid had strong oxidative force for alcohols, while they were very weak for sugars; they oxidized glucose but not gluconic acid, and lacked oxidizability for sugar-alcohols; sorbitol, mannitol, and glycerol. In contrast, the species isolated from the substances containing sugars, flowers and fruits, had strong oxidative activity for sugars and oxidized glucose and gluconic acid well. Moreover, they could also oxidize sugar-alcohols.

Besides, the former group characteristically oxidized acetic acid, and it also oxidized succinic, fumaric, malic, and pyruvic acids. And, the species belonging to the latter group could be distinguished by the intensity of their oxidative pattern for ketohexoses: fructose and sorbose. Three species (Nos. 9 to 11) had weaker activity for sugar-alcohols than the others, and could not oxidize ketohexoses. Six species (Nos. 12 to 17) had strong oxidative activity for fructose only and other three species (Nos. 18 to 20) could well oxidize both ketohexoses.

In addition to the above mentioned facts, it was observed that some species lack oxidizability for alcohols (No. 20) or glucose (No. 1), and that the pigment producing species could also be classified as whether they strongly oxidize maltose or not and also whether they oxidize strongly pentoses or not.

These facts point out that *Acetobacter* species can be classified in two groups according to their oxidizability for carbohydrates.

Group 1: 1. Does not oxidize gluconic acid, sorbitol, and mannitol.
2. Oxidizes acetic acid.
Group 2: 1. Oxidizes gluconic acid, sorbitol, and mannitol.
2. Does not oxidize acetic acid.
Sub-group i. Produces pigment in the media.
ii. Does not oxidize ketohexoses.
iii. Oxidizes only fructose among ketohexoses.
iv. Oxidizes fructose and sorbose.

SUMMARY

Many strains of genus *Acetobacter* were isolated and collected from the various habitats, and twenty strains were selected for present study. Three new species and one variant were detected.

The oxidizabilities of the resting cells for various substrates were measured by the manometric method. Thus, two groups were observed in the species.

Acknowledgement. The authors are grateful to Dr. Hideo Katagiri of Kyoto University, Dr. Kikichi Sato of Institute of Fermentation, and Dr. Toshinobu Asai of Tokyo University for their kind guidance and suggestions.

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13) T. Asai, *J. Agr. Chem. Soc. Japan*, **11**, 693 (1935).

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 6, p. 373~379, 1958]

Carbohydrate Metabolism by *Acetobacter* Species

Part II. Ketogenic Metabolism of Glucose

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The oxidative activities of resting cells of *Acetobacter* on glucose, gluconate, and ketogluconates were measured manometrically. Moreover, the effect of 2,4-dinitrophenol on the oxidation was examined and it was found that *Acetobacter* species could be classified into four types according to the degree of inhibiting concentration of this agent for each species, thus indicating that four metabolic systems exist in glucose oxidation by this genus, as recognized in the cultural experiment. Thus, it was pointed out that the kinds of metabolic patterns and the oxidative intermediates in glucose oxidation could be analogized by this method of investigation.

It has been observed by many investigators that *Acetobacter* species oxidizes glucose to gluconic acid or, moreover, to ketogluconic acids. In the previous paper[1], it was found that many species oxidize glucose and gluconic acid, while some of them scarcely or slightly oxidize them. Therefore, measurement and comparison of activities of these reactions by using the resting cells were undertaken. Heretofore, the oxidizability of the resting cells of *Acetobacter* species has scarcely been reported. In this paper, the oxidizabilities of twenty resting cells of *Acetobacter* species on glucose, gluconic, 2-ketogluconic, and 5-ketogluconic acids have been investigated in the mass with the purpose to make clear the whole view of initial oxidations in the oxidative metabolism of glucose by them. The results obtained about ketogenic metabolism in cultural experiments have also been confirmed manometrically by using 2,4-dinitrophenol as an inhibiting agent for oxidation.

EXPERIMENTAL AND RESULTS

The consumption of oxygen per mg of dried cells of twenty species for one hour on four substrates was manometrically measured at 30°C in air phase. And RQ for glucose was determined.

Preparation of cell suspension Each species maintained on koji extract-agar slant inoculated on 5 to 10 slants containing 2% glucose, 1% yeast extract, 0.8% calcium carbonate, and 2% agar, and previously incubated at 30°C for 2 days. The cells were mildly gathered up by loop and suspended homogeniously in distilled water. After agar impurities were removed through absorbent cotton, cells were collected centrifugally and further washed with water twice, followed by suspension in sterilized water. In its 1 ml, 5 to 10 mg dried cells were contained. Negligible autorespiration in the cell suspension of *Acetobacter* species was not observed.

Preparation of substrates Glucose for injection use and recrystallized potassium salts of gluconic, 2-ketogluconic, and 5-ketogluconic acids were used.

Contents in vessels:		
	Cell suspension	1.0 ml
	M/15 Phosphate buffer (pH 6.0)	1.5 ml
	6% Substrate aqueous solution	0.3 ml

Besides, 0.2 ml of 15% potassium hydroxide solution to measure the consumption of oxygen or 0.3 ml of 3 N sulfuric acid for the evolution of carbon dioxide was used.

As shown in Table I, the following facts were observed in comparison of Qo_2 for these substrates and RQ for gluose.

(1) *Acetobacter* species were divided into two groups

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[1] K. Kondô and M. Ameyama, This Bulletin, **22**, 369 (1958).

TABLE I. OXIDATION OF GLUCOSE, GLUCONATE, AND KETOGLUCONATES BY RESTING CELL OF *Acetobacter* SPECIES

Strain Name	Substrate No.	Q_{O_2} Glucose	Q_{O_2} K-Gluconate	Q_{O_2} K-2-Ketogluconate	Q_{O_2} K-5-Ketogluconate	RQ
A. ascendens	1	50	3	0	0	1.0
A. aceti	2	140	13	0	0	1.0
A. pasteurianum	3	200	80	0	0	1.0
A. kützingianum	4	50	10	0	0	1.0
A. rancens	5	110	40	0	0	1.0
A. melanogenum	6	500	284	269	71	0.8
A. rubiginosus	7	540	250	250	60	0.8
A. aurantium	8	490	214	260	70	0.8
A. acetosum	9	450	350	90	79	0.8
A. dioxyacetonicus	10	540	360	92	83	0.8
A. orleanens	11	535	290	180	130	0.8
A. cerinus	12	510	410	186	186	0.6
A. gluconicum	13	525	400	80	100	0.6
A. xylinum	14	560	350	80	90	0.6
A. industrium	15	345	150	40	35	0.6
A. oxydans	16	545	300	85	86	0.6
A. roseus	17	550	340	82	88	0.6
A. albidus	18	382	120	25	30	0.2
A. suboxydans	19	502	160	9	14	0.2
A. suboxydans α	20	504	100	7	13	0.2

by their degree oxidizability of glucose and gluconate, and it was found that the one having weak oxidizability could not oxidize ketogluconates while the other group revealed remarkable oxidizability attacking the keto-acids very strongly.

(2) RQ values for glucose with the former group of *Acetobacter* were 1.0 and with the latter group were below 1.0.

(3) The latter group was also characterized as follows: i, in the oxidizability of gluconate, there were also two distinguished sub-groups, strong and weak sub-groups; ii, this difference was also recognized in the oxidizability of ketogluconates; iii, RQ values for glucose with the strong sub-group were from 0.6 to 0.8 and those with the weak sub-group were 0.2.

(4) The oxidizability of 2-ketogluconate of pigment-producing species (Nos. 6 to 8) was stronger than other species and, as these species revealed nearly equal oxidizability of gluconate, it was therefore suggested that an enzyme oxidizing 2-ketogluconate further is contained in these species.

(5) All species, which produced 2-ketogluconate in cultural experiments, equally oxidized 2- and 5-ketogluconates, while the species that mainly produced 5-ketogluconate had little oxidizability for both. The results of the cultural experiments are shown in Tables II to V.

(6) It was probably suggested that the smaller RQ value represents the smaller velocity decarboxylation compared with the velocity of oxidation and therefore, much accumulation of metabolic intermediates is caused in the media. This probably coincides with the phenomena that in culture containing glucose as carbon source, *Acetobacter* mainly accumulate ketogluconate in most cases.

It was presumed as discussed above that there exist some different metabolic systems when *Acetobacter* oxidizes glucose. And, it was found that a classification of *Acetobacter* species might be possible from the above point of view.

Effect of 2, 4-dinitrophenol on oxidation of glucose, gluconate, and ketogluconates.

It has been well known that in the range between 10^{-4} and 10^{-5} mole 2, 4-dinitrophenol generally inhibits the oxidative phosphorylation of aerobic metabolism in animals, plants, and microorganisms. Also in *Acetobacter*, it has been recognized to be " the inhibition of assimila-

TABLE II. PRODUCTION OF GLUCONIC ACID BY *Acetobacter* SPECIES

(Produced from glucose, 10 g per 100 ml, without calcium carbonate, by stationary culture, at 30°C, 7 days.)

Strain No.	Acidity (ml of 0.1 N NaOH per 10 ml of medium)			Gluconic acid produced (g/100 ml) Calc. from		Gluconic acid to glucose (per cent)
	Total acid	Volatile acid	Non-volatile acid	Acidity	Ca-Gluconate	
1	0.0	0	0	0	0	0
2	6.4	0	6.4	1.25	1.0	10
3	3.0	0	3.0	0.58	0.3	3
4	4.6	0	4.6	0.90	0.6	6
5	10.3	0.3	10.0	1.96	1.5	15
6	30.4	0.4	30.0	5.88	6.2	62
7	50.0	1.0	49.0	9.60	9.5	95
8	32.8	0.8	32.0	6.27	6.3	63
9	34.4	0.4	34.0	6.66	6.8	68
10	53.6	1.5	52.1	10.00	9.8	98
11	53.6	1.6	52.0	10.00	9.8	98
12	46.1	0.3	45.8	8.97	9.0	90
13	52.0	1.1	50.9	9.97	9.7	97
14	53.6	1.5	52.1	10.00	9.6	96
15	48.2	1.0	47.2	9.25	9.0	90
16	48.4	0.5	47.9	9.37	9.5	95
17	52.6	1.2	51.4	10.00	9.7	97
18	33.4	0.8	32.6	6.38	7.5	75
19	46.0	0.5	45.5	8.91	8.6	86
20	49.8	1.3	48.5	9.50	9.7	97

TABLE III. PRODUCTION OF GLUCONATE AND KETOGLUCONATES BY *Acetobacter* SPECIES

(Produced from glucose, 5 g, in presence of calcium carbonate, 1.2 g, by shaking culture, at 30°C, 7 days.)

Species No.	Reducing power Cu mg/ml	Optical rotation	Ca dissolved mg/ml	Ca-Gluconate g/50 ml	Ca-5-Keto-gluconate g/50 ml	Ca-2-Keto-gluconate g/50 ml	Ca Salt of other reducing substances g/50 ml
1	11.16	+0.30	4.28	2.0	—	—	
2	8.88	+0.35	7.98	3.0	—	—	
3	27.0	+0.50	8.30	3.7	—	—	
4	22.15	+0.51	9.01	4.0	—	—	
5	16.32	+0.43	8.01	3.0	—	—	
6	55.2	−1.06	6.9		0.1	0.9	2.3
7	105.4	−3.09	6.72	2.0	—	1.6	
8	81.8	−3.51	6.9	0.7	—	1.5	
9	137.8	−8.06	7.48		—	4.0	0.1
10	91.78	−5.47	7.14		—	3.0	0.2
11	135.8	−6.67	8.26		—	4.0	0.2
12	34.4	−1.27	3.06		3.9	0.3	
13	123.9	−6.10	6.0	—	1.0	3.0	
14	57.0	−2.73	3.10	0.2	2.5	1.0	
15	88.68	−4.07	5.78	1.0	0.5	2.1	
16	97.8	−4.65	5.30		1.5	2.5	
17	132.0	−5.98	7.16	0.1	0.7	3.4	
18	40.0	−1.70	4.20	1.1	2.4	0.5	
19	96.8	−4.64	5.08		2.1	2.0	
20	36.78	−1.60	1.58		4.2	0.4	

TABLE IV. PRODUCTION OF GLUCONATE AND KETOGLUCONATES BY *Acetobacter* SPECIES

(Produced from glucose, 2.5 g, in presence of calcium carbonate, 0.6 g, by stationary culture, at 30°C, 25 days.)

Strain No.	Reducing power Cu mg/ml	Optical rotation	Ca dissolved mg/ml	Ca Gluconate g/50 ml	Ca-5-Keto-gluconate g/50 ml	Ca-2-Keto-gluconate g/50 ml	Ca salt of other reducing substances g/50 ml
1	33.49	+0.48	3.0	1.7	—	—	
2	34.0	+0.55	3.5	1.5	—	—	
3	11.2	+0.31	2.7	1.5	—	—	
4	10.6	+0.31	2.6	1.5	—	—	
5	31.7	+0.83	2.6	1.8	—	—	
6	54.0	−0.23	2.3		—	0.2	0.8
7	48.0	−0.92	3.5		—	0.3	1.5
8	45.07	−0.25	3.1	0.4	—	0.2	
9	65.4	−3.77	4.0	0.2	—	1.1	0.2
10	88.2	−3.73	3.58	0.2	—	1.4	
11	66.0	−2.95	2.96	0.4	—	0.7	
12	43.2	−2.66	3.2	0.5	0.1	0.2	
13	85.2	−3.36	2.95		1.0	1.0	
14	57.2	−2.59	3.3	0.5	0.05	0.6	
15	23.08	−0.70	2.8	0.1	0.9	0.2	
16	71.5	−2.92	2.5		0.2	0.9	
17	51.6	−2.38	2.2	0.5	0.6	0.2	
18	10.0	−0.24	1.69		2.1	—	
19	14.48	−0.30	1.50		2.0	—	
20	18.3	−0.19	1.85		2.3	—	

TABLE V. PRODUCTION OF KETOGLUCONATES FROM GLUCONATE BY *Acetobacter* SPECIES

(Produced from Ca-gluconate, 2.5 g, by shaking culture, at 30°C, 7 days.)

Strain No.	Ca-Gluconate residued g/50 ml	Ca dissolved mg/ml	Reducing power Cu mg/ml	Optical rotation	Ca-5-Keto-gluconate g/50 ml	Ca-2-Keto-gluconate g/50 ml	Ca salt of other reducing substances g/50 ml
1	2.5	4.6	—	+0.40	—	—	
2	2.5	4.6	1.0	+0.45	—	—	
3	2.5	4.6	—	+0.41	—	—	
4	2.4	4.5	—	+0.41	—	—	
5	2.5	4.6	1.3	+0.40	—	—	
6*	—	2.8	23.2	−0.70	0.1	0.2	1.0
7*	—	3.7	36.5	−1.49	—	0.4	1.0
8**	—	2.9	35.0	−1.08	—	0.3	0.7
9	—	3.9	38.4	−2.04	—	1.0	0.1
10	—	3.3	60.0	−2.15	—	1.0	0.1
11	—	3.9	49.64	−2.37	—	1.0	0.1
12	0.6	3.52	22.6	−1.61	0.2	0.2	
13	—	2.56	13.8	−1.07	1.1	0.2	
14	0.2	3.0	38.4	−1.94	0.2	0.6	
15	—	2.6	28.85	−1.07	0.8	0.3	
16	0.3	4.1	40.5	−1.83	0.1	0.7	
17	—	1.74	17.6	−0.87	1.2	0.2	
18	0.1	2.2	16.4	−1.06	0.5	0.2	
19	—	0.8	0.66	+0.35	2.0	—	
20	—	0.5	4.4	+0.32	2.2	—	

* Media colour dark reddish brown.
** Media colour brown.

tion” as stated by Clifton[2,3], namely when this agent is added to the reaction system, the consumption of oxygen increases in oxidation of carbohydrates by intact cells, however, when the agent is used in high concentraions, in opposition, consumption of oxygen rather decreases, since the oxidation of substrates, itself, is inhibited by this agent. It has been reported by King and his co-workers[4,5,6] that the oxidations of glycerol and sorbitol by *Acetobacter suboxydans* proceed as far as dihydroxyacetone and sorbose respectively, with the consumption of 0.5 mole of oxygen per mole, by the addition of 10^{-3} mole of this agent, and also by Katznelson and his co-workers[7] that oxidations of glucose, gluconic-, and 2-ketogluconic acids by *Acetobacter melanogenum* are inhibited by the same concentration, and these substrates are oxidized as far as 2, 5-diketogluconic acid with the consumption of oxygen of 1.5, 1.0, and 0.5 mole per mole respectively. The reason for this inhibition is probably due to the fact that phosphorylation takes part in reactions effected by the agent, or in the coupling reactions.

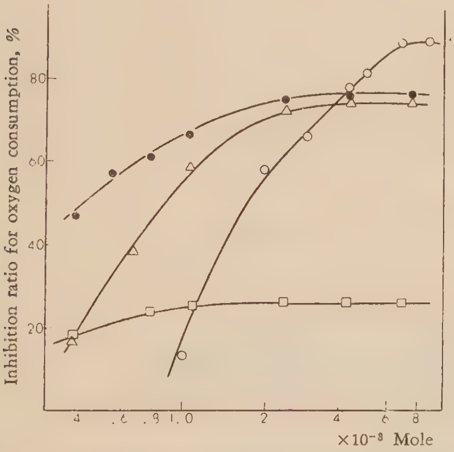

FIG. 1. Effect of 2, 4-Dinitrophenol on Oxidation of Glucose by Resting Cells of *Acetobacter* Species.

○, *A. pasteurianum*; □, *A. rubiginosus*; △, *A. dioxyacetonicus*; ●, *A. suboxydans* α.

The vessels contained 2.7 ml of cell suspension in M/15 phosphate buffer, pH 6.0, containing 2,4-dinitrophenol; 0.3 ml of glucose (2.5 μM); 0.2 ml of 15 per cent KOH in centre well; endogenous subtracted.

Then, the authors employed this method for studies on the oxidation system of glucose by *Acetobacter* species as follows. First of all, the effect of concentration of this agent was investigated. 2, 4-Dinitrophenol was dissolved in M/15 phosphate buffer, pH 6.42, and prepared as 10^{-2} mole solution. The effects of various concentrations of the agent for consumption of oxygen were observed using 2.5 μM glucose as a substrate. Thus, as shown in Fig. 1, twenty species were divided into four types from the above results. Therefore the necessary concentration for the maximum inhibition of the agent for each type, as shown in Table VI, was used in the following experiments. Whereupon, it was found that in oxidation of each 2.5 μM of glucose, gluconate, 2-ketogluconate, and 5-ketogluconate under the influence of known concentrations of the agent, each species belonging to the same type gave the same result. Fig. 2 sohws the results obtained in each typical species —Nos. 3, 7, 10, and 20, respectively, from Nos. 1 to 5, 6 to 8, 9 to 17, and 18 to 20. In this experiment autorespiration was subtracted since the duration of experiment was long and the concentration of the used substrates was low. Evolution of carbon dioxide was not recognized in any experiment within the whole duration.

TABLE VI. CLASSIFICATION OF *Acetobacter* SPECIES FROM EFFECT OF 2, 4-DINITROPHENOL UPON OXIDATION OF GLUCOSE BY RESTING CELL

Type	Strain No.	Max. inhibition ratio, %	Concentration of 2, 4-DNP necessary for max. inhibition
A	1~5	89	7×10^{-3}M
B	6~8	25	1
C	9~17	76	2
D	18~20	77	2

Corresponding to the results in the cultural experiment, the results shown in Fig. 2 were also very interesting. In the cultural experiment, it was observed that all species accumulated in the media either gluconate, 2-ketogluconate, and 5-ketogluconate, or both of the two ketogluconates as the main metabolic product of glucose, and that the pigment-producing species among 2-ketogluconate formers were different from the others since they produced only 2, 5-diketogluconic acid. The phenomena that these metabolites are accumulated in the media suggest that in the system of a series of reactions on glucose oxidation, the reaction, in which the accumulative metabolites are oxidized on step further,

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2) C. E. Clifton, *Enzymologia*, **4**, 246 (1937).

3) C. E. Clifton, *Adv. Enz.*, **6**, 269 (1946).

4) T. E. King and V. H. Cheldelin, *Science*, **115**, 4 (1952).

5) T. E. King and V. H. Cheldelin, *J. Biol. Chem.*, **198**, 135 (1952).

6) T. E. King and V. H. Cheldelin, *J. Bacteriol.*, **66**, 581 (1953).

7) H. Katznelson, S. W. Tanenbaum and E. L. Tatum, *J. Biol. Chem.*, **204**, 43 (1953).

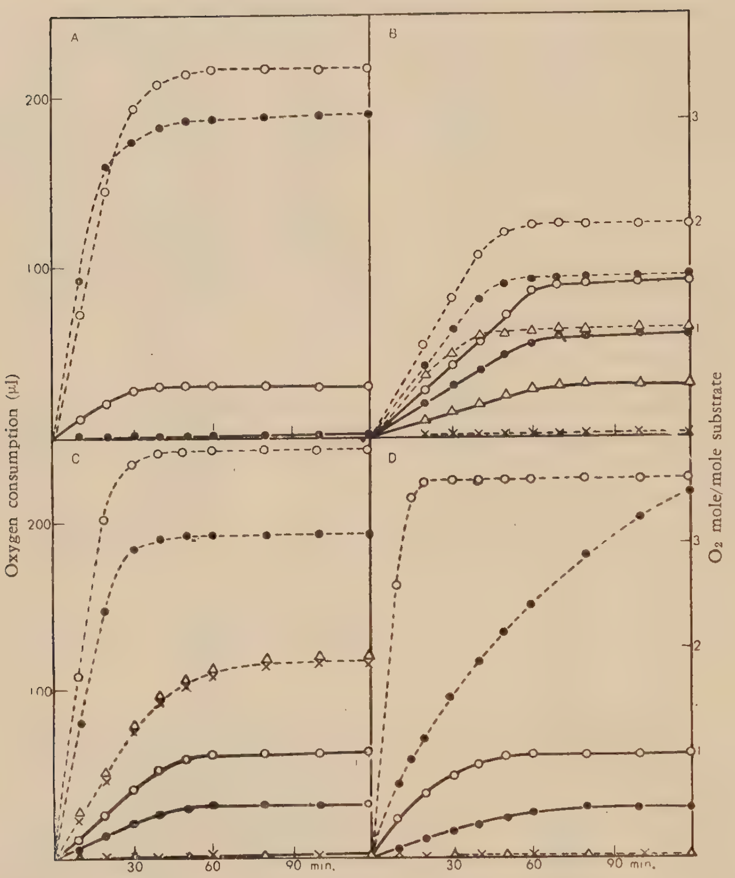

FIG. 2. Oxidation of Glucose, Gluconate, and Ketogluconates by Resting Cells of *Acetobacter* Species, as influcenced by 2, 4-dinitophenol.

A, *A. pasteurianum*; B, *A. rubiginosus*; C, *A. dioxyacetonicus*; D, *A. suboxydans α*.
The vessls contained 2.7 ml of cell suspension in M/15 phosphate buffer, pH 6.0; 0.3 ml of glucose (○), K-gluconate (●), K-2-ketogluconate (△), and K-5-ketogluconate (×), each 2.5 μM; 0.2 ml of 15% KOH in centre well; endogeneous respiration was subtracted. Solid line; with 2,4-dinitrophenol. Dash line; without the agent.

is probably different from former oxidative reactions for some reason, and that 2, 4-dinitrophenol affects this enzyme reaction especially. Thus these results obviously show that, as it is seen in Table VII, there are four distinct different processes in oxidative metabolism of glucose by *Acetobacter* species, as follows: as influenced by 2, 4-dinitrophenol, a process in which oxidation of glucose proceeded as far as gluconate but not further (Fig. 2-A), one in which glucose oxidation proceeded as far as ketogluconates but not further and, among this process there were further two types in the absence of the agent, i. e., in one type ketogluconates were oxidized while in the other type they were not—(C and D), and one in which oxidation proceeded as far as 2, 5-diketogluconate (B). Besides these, in the last process, as shown in Fig. 2-B, 2-ketogluconate was oxidized as far as diketogluconate, but 5-ketogluconate was not completely attacked. In other words, it has been found that the metabolic intermediates, which pass through or accumulate in incubation starting from glucose as carbon source, can be presumed from such experiments to be influenced by this agent, and that a metabolic pattern of glucose or an analogy with a known species by newly isolated *Acetobacter* can be made

TABLE VII. EFFECT OF 2, 4-DINITROPHENOL UPON OXIDATION OF GLUCOSE BY RESTING *Acetobacter* SPECIES

Type	Moles oxygen consumed per mole of			Assuming accumulative intermediate
	Glucose	Gluconate	Ketogluconate	
A	0.5	0	0	Gluconate
B	1.5	1.0	0.5	2, 5-Diketogluconate
C	1.0	0.5	0	Ketogluconate
D	1.0	0.5	0	Ketogluconate

clear by applying this method of investigation.

Effect of pH on oxidations of glucose, gluconate, and ketogluconates.

There are probably one enzyme producing 2-ketogluconic acid and another one producing 5-ketogluconic acid from gluconic acid. However, it was observed of course in species forming both ketogluconic acids, even in ones forming single ketogluconic acid especially that the produced quantities of each ketogluconic acids are varied by the cultural pH, and interestingly that 5-ketogluconate former grew well in acidic environment than 2-ketogluconate former. Therefore, the authors have investigated the effect of pH on the production of two ketogluconic acids and the enzyme reaction on the oxidation, using cell suspension of strain No. 10 as a 2-ketogluconate former and strain No. 20 as a 5-ketogluconate former.

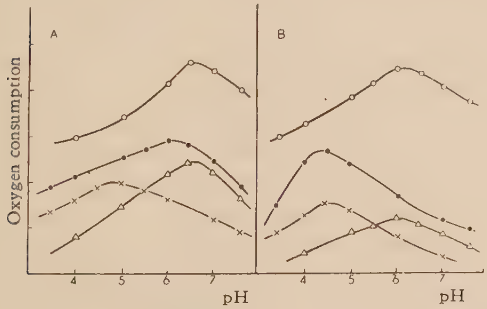

FIG. 3. pH Effect upon Oxidation of Glucose, Gluconate, and Ketogluconates by Resting Cells of 2-Ketogluconate Former and 5-Ketogluconate Former of *Acetobacter* Species.

A, *A. dioxyacetonicus*; B, *A. suboxydans α*.

The vessels contained 1.0 ml. of cell suspension in water; 1.5 ml of M/15 phosphate buffer; 0.3 ml of glucose (○), K-gluconate (●), K-2-ketogluconate (△), and K-5-ketogluconate (×), each 100 μM; 0.2 ml of 15 per cent KOH in centre well; endogeneous not subtracted.

Cell suspensions were prepared as mentioned above. Oxygen consumed was measured for glucose, K-gluconate, K-2-ketogluconate, and K-5-ketogluconate, respectively at 30°C. Content of Warburg's vessels were composed of 1.0 ml of cell suspension, 1.5 ml of M/15 phosphate buffer, 0.3 ml of 6% susbstrate aqueous solutions, and 0.2 ml of 15% potassium hydroxide solution. As shown in Fig. 3 and Table VIII, each optimum pH on oxidation of the four substrates were as follows; i, optimum pH with both species were similarly between 6 and 7 for oxidations of glucose and 2-ketogluconate; ii, with both spcies optimum pH were between 4 and 5 for 5-ketogluconate; and iii, for gluconate a distinct difference was recognized between the two species, namely with the 5-ketogluconate former optimum pH was found to be pH 4.2[8] while with the 2-ketogluconate former it was pH 6.2.

TABLE VIII. OPTIMUM pH UPON OXIDATION OF GLUCOSE, GLUCONATE, AND KETOGLUCONATES BY *Acetobacter* SPECIES

Substrate / Strain	Glucose	Gluconate	2-Ketogluconate	5-Ketogluconate
2-Ketogluconate former	6.6	6.2	6.5	4.8
5-Ketogluconate former	6.1	4.2	6.1	4.5

SUMMARY

(1) The oxidative activities on glucose, gluconate, 2-ketogluconate, and 5-ketogluconate by resting cells of *Acetobacter* were measured manometrically.

(2) The effect of 2, 4-dinitrophenol on oxidations was observed and it was found that *Acetobacter* species is classified into four types.

(3) The effect of pH on the oxidation was observed and discussed.

Acknowledgement. The authors are grateful to Dr. Hideo Katagiri of Kyoto University for his kind guidance and suggestions.

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[8] M. Yamazaki, *J. Agr. Chem. Soc. Japan*, **28**, 748 (1954).

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 6, p. 380~386, 1958]

Carbohydrate Metabolism by *Acetobacter* Species

Part III. Isolation and Identification of D-Lyxuronic Acid on Glucose Oxidation by *A. melanogenum*

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Received May 19, 1958

Development of oxidation products from glucose by growing *A. melanogenum* was observed. A penturonic acid-like compound, one of the main products, was isolated and identified with D-lyxuronic acid. D-Lyxuronic acid was isolated as crystallized and lyophilized calcium salt. $[\alpha]^{20}_{D}$ of the calcium salt was −23°, and showed mutarotation. It had a furanose ring structure in solid phase. It consumed three moles of periodate per mole and was then divided into glyoxylic and formic acids. It was discussed that D-lyxuronic acid was produced from 2,5-diketogluconic acid, with decarboxylation of the first carbon, via probably 4-ketoarabinose and 4-ketoarabonic acid. In the oxidative products, phosphate ester compounds were not detected.

As mentioned in the previous paper, it was observed that the pigment-producing species produced a large amount of unknown strong reducing organic substances other than 2-ketogluconic acid from glucose or gluconic acid. As ketogluconic acid originally ought to be oxidized further and consumed by cells, it was thought that these unknown reducing substances were probably oxides of ketogluconic acid in the later period. This fact was realized by the isolation of 2,5-diketogluconic acid, as reported by Katznelson and his co-workers[1], using the resting *A. melanogenum*, by K. Aida *et al.*[2] using *Gluconobacter liquefaciens*, and by the present authors[3] using the growing cells of *A. melanogenum*.

In the latter authors' report[3], it was observed additionally that an unknown penturonic acid-like compound is produced in the glucose media. Besides, Katznelson *et al.* reported[4] that the cell-free enzyme preparation produced a "pentose-like compound" from 2,5-diketogluconate anaerobically, with liberation of carbon dioxide, and, when the reaction mixture was exposed to air and phenazine methosulphate was added, this pentose-like compound disappeared with the concomitant formation of α-ketoglutarate.

In this paper, isolation of the unknown penturonic acid, "pentose-like compound", and α-ketoglutarate, mentioned above, was undertaken in growing culture of *A. melanogenum*. As a result, two reductones, penturonic acid, and two or three dicarbonyl-pentoses were detected as new oxidation products of glucose. But no α-ketoglutaric acid was found. This penturonic acid was then identified with D-lyxuronic acid.

EXPERIMENTAL AND RESULTS

A. melanogenum, previously incubated for two days in 10 ml media containing 0.8% yeast extract, 10% glucose, and 2.5% calcium carbonate, was grown at 30°C in 100 ml media, under the shaking condition, and in 250 ml, under the static one, composed of the same substances and distributed in 500 ml flasks. While the metabolic products were separated, the cultural development was studied paper chromatographically. Isolation and identification of most metabolic products are mentioned previously[3].

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[1] H. Katznelson, S. W. Tanenbaum, and E. L. Tatum, *J. Biol. Chem.*, **204**, 43 (1953).

[2] K. Aida, M. Fujii, and T. Asai, *Proceedings of the Japan Academy*, **32**, 595 (1956).

[3] K. Kondô, M. Ameyama, and T. Yamaguchi, *J. Agr. Chem. Soc. Japan*, **30**, 419, 423 (1956); *C. A.*, **51**, 18104 c.

[4] A. G. Datta and H. Katznelson, *Nature*, **179**, 153 (1957)

Fermentative broth, removed of cells and residual calcium carbonate with the aid of diatom, was subsequently put through an IR 120 (H^+) resin column to remove dissolved calcium ions completely, followed by concentration in vacuo below 30°C by gassing with carbon monooxide. This was then shaken with ether a few times in a distributing funnel and divided out from the ether-soluble part. In this ether-soluble part two reductones were detected with rubiginol. The other part was further extracted continuously with ether for four to six days in a Soxlet extractor. The ether-soluble part contained glycolic-, tartronic-, comenic-, rubiginic acids, and rubiginol, and also oxalic- and succinic acids when the media were incubated for a long period. Two reductones could be detected as pink spots with phloroglucin-hydrochloric acid, as the developing agent. And, of course, these were intensively positive for ammoniacal silver-nitrate, indophenol, and brom phenol blue. When a fraction containing them was neutralized with sodium bicarbonate and divided out of rubiginol by means of another extraction, the solution consisting of only two reductones instantaneously showed black color by addition of ferric chloride solution. Ether-insoluble part was neutralized with calcium carbonate and decolored with the aid of active carbon. Calcium salts of gluconic, 2-ketogluconic, and 2, 5-diketogluconic acids, and penturonic acid-like and other sugar compounds were distributed in this filtrate.

The paper chromatogram of broth obtained under the static condition is shown in Fig. 1, which showed an interesting development of products as follows: first of all, gluconic and 2-ketogluconic acids were produced, followed by production of 2, 5-diketogluconic and penturonic acids, succeeded by the accumulation of tartronic, glycolic, and succinic acids and the production of two reductones and 4-pyrone derivatives at the same time. Oxalic acid appearing after one month increased gradually with glycolic and succinic acids, but all other acids disappeared. Pyruvic and α-ketoglutaric acids were not detected in this experiment. Further, no evidence of phosphate esters was found in the previous products by tests with molybdate reagent.

Isolation of penturonic acid-like compound. The fermentative broth, incubated from thirty to forty days under the static condition, was decolored with the aid of lead sulfide and active carbon, followed by concentration to syrup, and added with ethanol up to 50%. From the supernatant, the calcium salt was precipitated

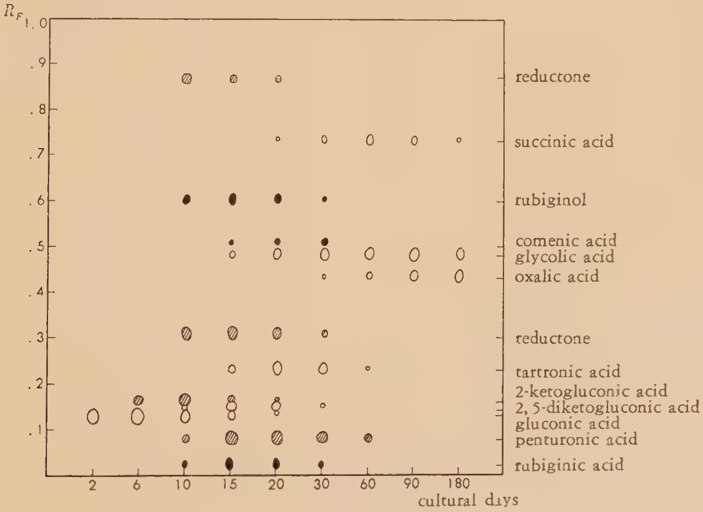


FIG. 1. Chromatographical Development of Glucose Oxidation by *A. melanogenum* (under static condition).

○, BPB; ▨, BPB and phloroglucin-HCl; ●, ferric ion positive.
(n-BuOH : HCOOH : H_2O=4 : 1.5 : 1)

by 80% ethanol. Its aqueous solution consisted of ether-soluble compounds, mainly consisting of glycolic acid, and penturonic acid-like compounds detected chromatographically. (The supernatant by 80% ethanol mainly contained pentose-like compounds.) This solution was put through and IR 120 (H^+) resin column to remove dissolved calcium ions, followed by ether extraction to remove glycolic acid and other ether-soluble compounds. The ether-insoluble solution was then fractionated to acidic and non-acidic parts by passing through an IR 4B ($CO_3^{=}$) resin column. The acidic part was eluted by 0.1 N sodium carbonate solution.

The non-acidic fraction consisted of three sugars, which revealed spots of R_F 0.55, 0.40, and 0.25 on paper partition chromatogram, developed ascendingly by employing *tert*-BuOH-formic acid-water (4 : 1 : 1.5) solvent. As shown in Table I, they were considered to be pentose-like compounds, but other than common pentoses. Besides, in the acidic fraction, after passing through an IR 120 resin column, only one acid was detected, which had a spot of R_F 0.40. (If, other ether-insoluble acids rarely happen to exist concomitantly, they can easily be removed by reprecipitation of calcium salts with ethanol.)

TABLE I. CHROMATOGRAM OF FERMENTATIVE BROTH

(30 to 40 culture days, under static condition. After decolorlization, ether-insoluble part.)

Fraction	R_F	Color with	
		aniline-oxalate	phloroglucin-HCl
Acidic	0.40	pink	blue
Non-acidic	0.55	pink	blue
	0.40	(pink)-brown	green
	0.25	brown	blue-green

Identification with D-lyxuronic acid. Calcium salt of penturonic acid-like compound isolated as mentioned above was completely decolored, followed by lyophilization, and identified with calcium salt of D-lyxuronic acid. Though the prismatic crystals of the calcium salt were obtained in acetone solution after a few months (Fig. 2) the amount was not adequate. The lyophilized calcium salt yield was about 5 g from 2 l of broth.

The salt showed typical pentose reactions with orcin- and phloroglucin-hydrochloric acid and aniline-acetic acid paper tests. Moreover, it showed typical uronic acid reactions with naphthoresorcin-hydrochloric acid

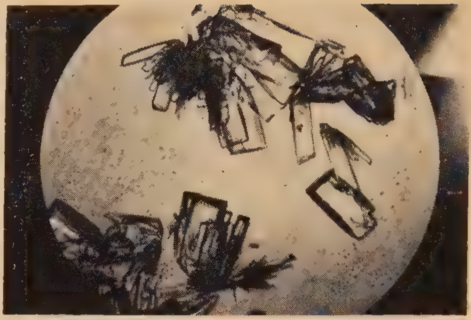


FIG. 2. Crystals of Ca-D-Lyxuronate Furanoside.

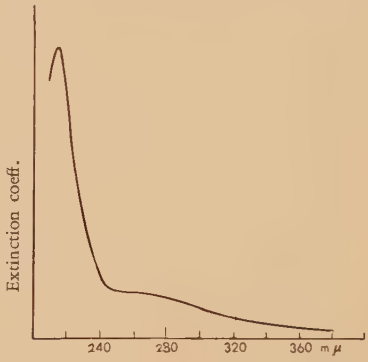


FIG. 3. Spectrum of D-Lyxuronate (pH 6.3).

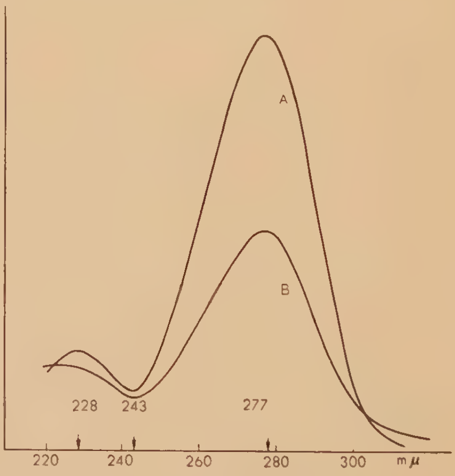
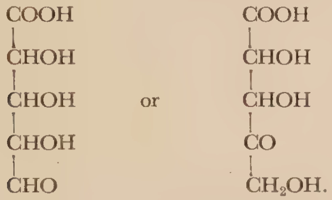


FIG. 4. Spectra of Distilate obtained by Decomposition with Hydrochloric Acid.

A : xylose, B : penturonic acid-like compound.

and carbazol-sulfuric acid tests. It strongly reduced ammoniacal silver nitrate and Benedict's reagents, and also produced crystals with semicarbazine and phenylhydrazine. As shown in Fig. 3, its spectrum showed the absorption of a carboxyl radical and a weak absorption of carbonyl group (260 to 270 mμ) (pH 6.3). When it was boiled with 12% hydrochloric acid, the distillate had an absorption spectrum as shown in Fig. 4. This spectrum did not have a maximum in 228 mμ differing from common pentoses.

The salt contained 15.25% crystal water, 27.45% C, 5.09% H, and 10.79% Ca. Therefore, it agreed with $C_5H_7O_6 \cdot Ca/2 \cdot 2H_2O$: calculated crystal water 16.44%, 27.40% C, 5.06% H, and 9.14% Ca. From the above qualitative data and formula, its structure, freed from calcium, was considered as follows:

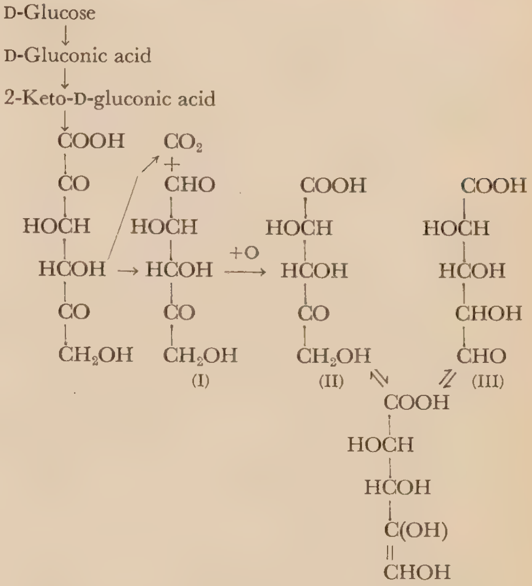


$$\begin{array}{c} COOH \\ | \\ CHOH \\ | \\ CHOH \\ | \\ CHOH \\ | \\ CHO \end{array} \quad \text{or} \quad \begin{array}{c} COOH \\ | \\ CHOH \\ | \\ CHOH \\ | \\ CO \\ | \\ CH_2OH. \end{array}$$

Although, in the latter formula, one of the three probable positions of the carbonyl group was considered to be in the second, third, or fourth carbon, it is probably in the fourth due to the following reasons:

It was suggested by the experiments of Katznelson et al.[1] that "such organisms may carry out a further single step oxidation of the diketogluconic acid to a ketopentonic acid", with the production of carbon dioxide. This fact was also supported by Aida et al.[5] on the basis of their experiment using 1-C*-gluconate as carbon source, liberated carbon dioxide had radioactivity.

Both of the above C_5 compounds can reciprocally change in the presence of isomerizing enzyme. Therefore these compounds are probably produced from glucose in the following way, so that the configuration on the second and third carbons are decided as shown follows:



Besides, $[\alpha]_D^{20}$ of the calcium salt was −23° and changed to −53° after thirty minutes. This mutarotation suggested that it could have a ring structure. Furthermore, an infra-red spectrum of its solid phase showed absorptions (803, 870, and 925 cm^{-1}) of a typical furanose ring[6], though typical carbonyl absorption (ca. 1700 cm^{-1}) has not been observed, as shown in Fig. 5. These facts suggested that the unknown compound was the salt of uronic acid (III), which could have furanose-ring structure, though compound (II) could not have such structure.

Periodate oxidation. It was recognized by periodic oxidation that the penturonic acid-like compound, freed from calcium, was either (II) or (III), rather (III). Twenty and a half miligram of the calcium salt in 10 ml of water was treated in an ice jar with 150 mg of sodium periodate in 10 ml water. Samples were withdrawn from the reaction mixture at intervals of five to ten minutes; liberated iodine contained in the mixture was treated by constant quantities of 0.01 N sodium arsenite in the presence of sodium bicarbonate, and excess arsenite was titrated with 0.01 N iodine solution. Consequently, consumption of three moles of periodate per mole was observed. And also, the residual reaction mixture after being analyzed for one night by ascending chromatography indicated the presence of glyoxylic and formic acids, besides iodic and periodic

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[5] K. Aida, M. Fujii, and T. Asai, Reported at the annual meeting of the Agricultural Chemical Society of Japan, held in 1957.

[6] S. A. Barker and R. Stephens, *J. Chem. Soc.*, **1954**, 4551.

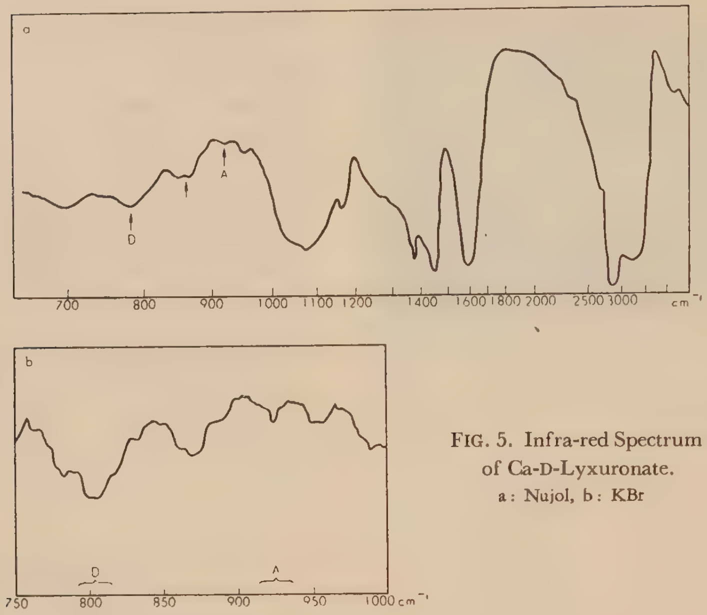


FIG. 5. Infra-red Spectrum of Ca-D-Lyxuronate.
a: Nujol, b: KBr

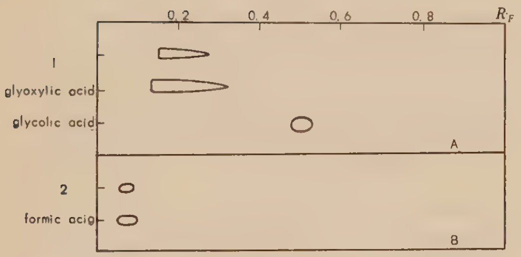


FIG. 6. Periodate Oxidation of D-Lyxuronic Acid. (at 0°C)

1: ether-soluble part of reaction mixture.
2: distillate of reaction mixture.
A: solvent (n-BuOH : HCOOH : H_2O=4 : 2 : 0.5)
B: solvent (3% NH_4OH saturated EtOH)

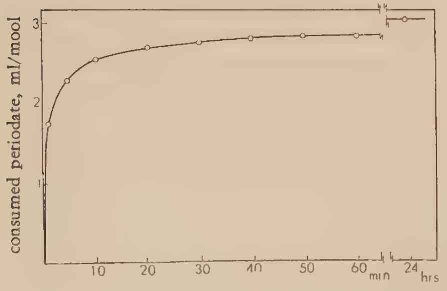
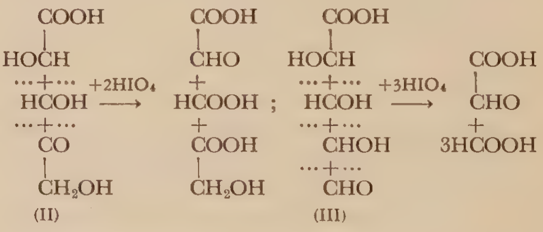


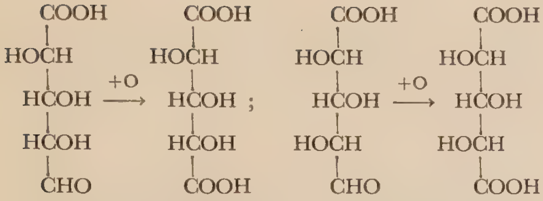
FIG. 7. Periodate Oxidation of D-Lyxuronic Acid. (at 0°C)

acids. Formic acid was detected by distillation and formation of mercurous chloride with mercuric chloride. Further, glyoxylic acid was also found by ether extract, and its 2, 4-dinitrophenylhydrazone was identified with an authentic derivative of glyoxylic acid, made from oxalic acid by reduction with magnesium, which revealed the spot of R_F 0.23 ascendingly by employing 3% ammonium hydroxide saturated n-BuOH solvent, and melted at 202°C. These results, shown in Figs. 6 and 7, pointed out that the structure of the unknown compound was (III), illustrated as follows:



(II) (III)

Bromine oxidation. The result previously mentioned indicates that the penturonic acid-like compound had the structure of (III). Namely, it was either D-lyxuronic or D-xyluronic acid. Then, when it was oxidized with bromine, the production of either D-arabo-trihydroxyglutaric or inactive xylo-trihydroxy-glutaric acid was expected, as follows:



D-lyxuronic acid D-arabo-trihydroxy-glutaric acid D-xyluronic acid inactive xylo-trihydroxy-glutaric acid

One gram of the calcium salt was treated with 50 ml of water, 10 g of calcium carbonate, and 1.5 g of bromine for two days at room temperature. After the removal of excess bromine and calcium carbonate by heating and filtration respectively, the solution was put through an IR 120 (H^+) resin column to remove dissolved calcium ions, followed by concentration to syrup. After several days it crystallized out in a refrigerator. This was easily recrystallized from acetone, and colorless crystals melting at a range from 126° to 128° and showing $[\alpha]^{30}_{D}+23°$ were obtained. These data agreed with those of the authentic D-arabo-trihydroxy-glutaric acid prepared from D-fructose[7,8]. The melting point of the mixture was also from 126° to 128°. Besides this, authentic xylo-trihydroxy-glutaric acid, prepared from D-xylose[9,10], melted at 152° and showed no rotation.

DISCUSSION

(1) As mentioned above, a penturonic acid-like compound was isolated from glucose media in which *A. melanogenum* grew, and identified with D-lyxuronic acid. This compound is a rarity which has never been reported again since Bergmann in 1921[11], synthesized DL-lyxuronic acid from DL-mucic acid, passing through DL-mucic monoamide, tetraacetyl-lyxuronamide, and lyxuronamide osamine in order. This method of Bergmann was not only very intricate and inefficient, but also never proved to be successful in the separation of free acid or its salts in crystal-form. Also as only the D-form of lyxuronic acid could not be synthesized by the authors after applying various methods, a dicarboxylic acid produced from penturonic acid-like compound by bromine oxidation was identified with D-arabo-trihydroxy-glutaric acid.

(2) D-Lyxuronic acid was isolated as the main oxidative metabolites of glucose, as mentioned above. Therefore, the authors have assumed that D-lyxuronic acid is produced from D-glucose via 2,5-diketogluconic acid and probably via "4-ketoarabinose" (I) and "4-ketoarabonic acid" (II). Two spots showing R_F 0.40 and 0.55 in the non-acidic fraction shown in Table I have been presumed as 4-ketoarabinose, which is produced by decarboxylation from carboxyl group of diketogluconic acid as previously mentioned[1,5], and its equilibrating sugar "dialdose" (I′). Such organism may have isomerase for these dicarbonyl-sugars, as well as the existence of isomerase for D-lyxuronic acid as suggested from the above result.

Next, metabolites are probably produced from "4-ketoarabonic acid" but not from D-lyxuronic acid, because (i) D-lyxuronic acid is accumulated in the media remarkably, despite that C_6-dicarboxylic acid, as an isomer 2,5-diketogluconic acid, is not detected, and (ii) a great deal of glycolic acid is produced in the media (10% of consumed glucose) and this acid may be formed from the fourth and fifth carbons of 4-ketoarabonic acid, for in experiments using 1-C*-gluconate as carbon source glycolic acid has no radioactivity[5].

The metabolic pathway from glucose by *A. melanogenum*, is therefore probably represented as follows:

Besides, two reductones in the ether-soluble fraction were thought to be produced from dicarbonyl sugar (I) or (I′) and 4-ketoarabonic acid (II), by dehydration.

(3) Also, by other pigment-producing *Acetobacter*, *A. rubiginosus* and *A. aurantium*[12], the same products from glucose were detected.

(4) As seen from the above results (and

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[7] Rao, Göttinger Dissertation, 1912.

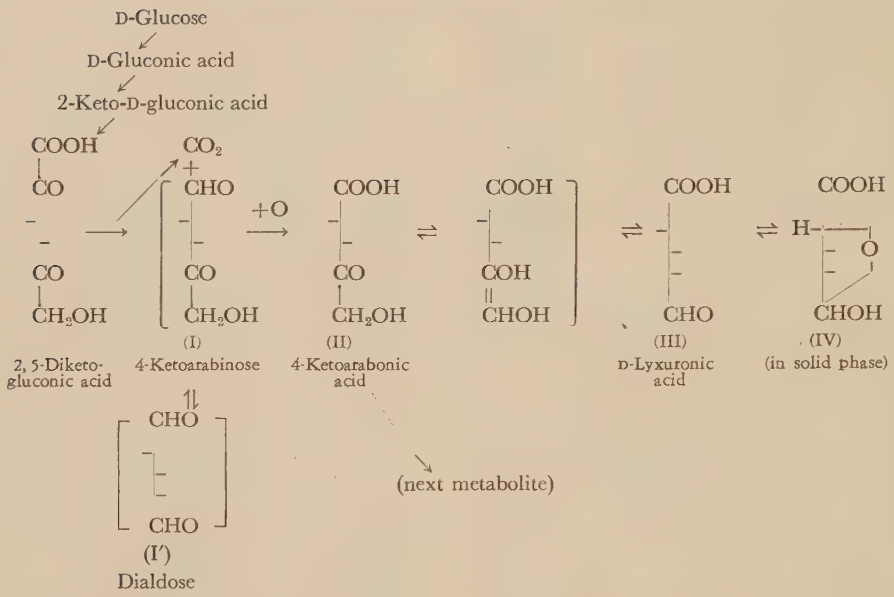
[8] E. v. Lippmann, *Ber.*, **26**, 3059 (1893); **32**, 1213 (1899).

[9] E. Fischer, *Ber.*, **24**, 1842 (1891).

[10] Wheeler and Tollens, *Ann. Chem.*, **254**, 318 (1889).

[11] M. Bergmann, *Ber.*, **54B**, 1362 (1921); *C. A.*, **15**, 3624 (1921).

[12] K. Kondô and M. Ameyama, This Bulletin, **22**, 369 (1958).



furthermore in the author's previous papers on *Acetobacter*), phosphate esters have hitherto never been detected, despite the fact that they have been detected in glucose oxidative metabolism by other aerobic microorganisms. Therefore, it was suggested that *Acetobacter* species have a new metabolic system differing from other microorganisms, concerning the oxidation of glucose.

Acknowledgement. The authors wish to express their hearty thanks to Dr. Katagiri of Kyoto University for his kind guidance and encouragement, and are also indebted to Dr. Ashida, Dr. Tamura, Mr. Kuyama of Nagoya University, Dr. Machida of Kyoto University of Industrial Arts & Textile Fibers, Dr. Ogata of Institute of Fermentation, and Mr. Kamiya of Shizuoka University for their kind suggestions and helpful comments.

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Effect of Some Purines on the Nitrate Reduction of Mouse Liver

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Received May 19, 1958

The nitrate reduction of the mouse liver homogenate was inhibited by several purines which have free-N at the positions of 1, 3, and 7, such as adenine, guanine, hypoxanthine, xanthine, 6-mercaptopurine, 8-azaguanine and 8-azaxanthine. Since the liver contains xanthine oxidase, the decoloration of methylene blue was promoted by hypoxanthine and xanthine, and it was delayed by other purines. On the contrary, caffeine and theophylline, N-methyl derivatives of purine were not effective or less effective in inhibiting the reduction of methylene blue as well as nitrate. The relation between nitrate reduction and the xanthine oxidase was presumed.

Formerly, it was demonstrated by Bernheim and Dixon[1] that the aldehyde oxidase of liver is able to reduce nitrate which can simply serve as a hydrogen acceptor in place of methylene blue like in case of the milk xanthine oxidase[2]. Several years ago, however, Egami and coworkers[3] emphasized that the participation of the aldehyde oxidase in the nitrate reduction of liver should be reviewed, since the reduction was exceedingly inhibited by acetaldehyde, whereas the decoloration of methylene blue had been accerelated. Recently, Westerfeld et al.[4] Summarized that methylene blue and nitrate can function as a hydrogen acceptor of the xanthine and aldehyde oxidases of several sources. It is supposed, therefore, that these enzymes might have an intimate connection with the nitrate reduction, though the mechanism could not be elucidated. On the other hand, the conjunction of the nitrate reduction with some dehydrogenases was also suggested[5].

In the previous paper[6], it was reported that silkworm tissue contains an inhibitor of the nitrate reduction of animal liver and green algae. This inhibitor was isolated and identified with uric acid, the main nitrogenous substance in the excrements of insect. Furthermore, it was found that the decoloration of methylene blue which was settled as an index of the ability of the hydrogen transfer was also retarded by the same compound.

Uric acid (2,6,8-trioxypurine) is a catabolic product of nucleic acid by way of the constituting amino purines, adenine (6-aminopurine) and guanine (2-amino-6-oxypurine), and their deaminated compounds, hypoxanthine (6-oxypurine) and xanthine (2,6-dioxypurine). In addition, the acid may also be formed as the end-product of the protein degradation and excreted in uricoteric metabolism such as insect and bird. On the other hand, in ureoterite, e.g. mammalia, uric acid is converted to urea, which has no inhibitory effect on the uitrate reduction, by the catabolic action of uricase system. In fact, it was observed that the inhibition by uric acid was depressed when it had been worked with the mouse liver homogenate containing the uricase

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[1] F. Bernheim and M. Dixon, *Biochem. J.*, **22**, 125 (1928).

[2] M. Dixon and S. Thurlow, *Biochem. J.*, **18**, 989 (1924).

[3] F. Egami, S. Suzuki, M. Niwa and R. Sato, *J. Chem. Soc. Japan*, **71**, 226 (1950).

[4] W. W. Westerfeld, D.A. Richert and E.S. Higgins, "A Symposium on Inorganic Nitrogen Metabolism", edit. by W.D. McElroy and G. Glass, The Johns Hopkins Press, Baltimore, 1956, p. 429.

[5] H. Omura, *Sci. Bull. Fac. Agr Kyushu Univ.*, **14**, 423 (1954).

[6] H. Omura, This Bulletin, **21**, 126 (1957)

TABLE I. EFFECT OF PHYSIOLOGICAL PURINES ON THE NITRATE REDUCTION AND THE DECOLORATION OF METHYLENE BLUE

Conc. of purine added, M		0	10^{-3}	5×10^{-4}	10^{-4}	5×10^{-5}	10^{-5}
Adenine	NO_2 formed, μM	34.0	2.9	3.2	5.7	8.3	18.9
	Inhibition, %		91.5	90.6	83.2	75.3	44.4
	M.B. decol. time, min.	3	10	9	7	5	4
Guanine	NO_2 formed, μM	26.2		13.0	17.2	20.0	25.2
	Inhibition, %			50.4	34.4	23.3	3.8
	M.B. decol. time, min.	7		16	11	10	8
Hypoxanthine	NO_2 formed, μM	33.6	7.4	7.7	15.4	21.2	32.0
	Inhibition, %		78.0	77.1	54.2	31.9	4.8
	M.B. decol. time, min.	48	22	18	16		
Xanthine	NO_2 formed, μM	32.0	4.0	4.7	13.7		
	Inhibition, %		87.5	85.1	58.7		
	M.B. decol. time, min.	57	38	31	26		

system prior to nitrate reduction, while it was kept constantly in case of the fowl liver which lacks in uricase. Hereupon, the influence of the physiological purines, the precoursors of uric acid, and some substituted analogues were successively investigated.

EXPERIMENTS AND RESULTS

Investigations were carried out with the mouse liver homogenate according to the usual manner[7], except that the homogenate was prepared as follows. Soon after killing mice by decapitation, the washed livers were homogenized with 10 vol. of M/10 phosphate buffer of pH 6.0 by a glass homogenizer of the Elvehjem type, driven by a small motor and centrifuged subsequently at 3000 r.p.m., for 5 min. The supernatant was filled up to a concentration of 3 g fresh liver per 50 ml with the same buffer. On each test, 5 ml of the homogenate thus made, were employed as an enzyme preparation. The decoloration of methylene blue was estimated by the procedure described in the foregoing paper[6].

As in the case with uric acid, it was preliminary examined whether or not the disturbance of the colorimetric estimation and the disappearance of the reduction product, nitrite, had been brought about by purines. In particular, cautious attention was paid to guanine and adenine, since these purines are deaminated chemically by nitrite in acidic media resulting in the decrease of nitrite, as described in a textbook. It was a matter of course that the experimental conditions were identical with those of the nitrate reduction. If such concern are to be realized, one must be prudent in explanation of the results in order to avoid misinterpreting due to the outward effect. However, neither detectable significant decrease nor the hindrance of the assay of nitrite was verified under the mild physiological conditions applied. Therefore, if the depression of the nitrate reduction had been recognized, it should be attributed to the net inhibition.

Effect of Natural Purines. The effect of the above-mentioned four physiological purines on the nitrate reduction and the decoloration of methylene blue is presented in Table I. Respective test was carried out using the homogenate prepared in a lump, since its activity varies with the materials. Concerning two amino purines, the inhibition of both activities was noticed as with uric acid. By hypoxantine and xanthine, on the other hand, nitrite production was exceedingly suppressed, while the decoloration of dye was conversely accerelated since the mouse liver contains not only several dehydrogenases but also xanthine oxidase. Furthermore, it was observed that the promotion of the enzymatic bleaching of dye was decreased as the amounts of substrate supplied was increased, as reported previously by Dixon and Thurlow[8] for milk xanthine oxidase. They emphasized even qualitatively, that xanthine oxidase can reduce nitrate and that substance inhibiting the enzymatic activity would also impede the nitrate reduction, and described the parallelism in the inhibitory effect of uric acid between the reductions of nitrate and methylene blue. Regarding the mouse liver enzyme, however, such correspondence with hypoxanthine xanthine could not be recognized, although it was supposed that the hindrance of the

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7) H. Omura, *J. Fac. Agr. Kyushu Univ.*, **10**, 365 (1956).

8) M. Dixon and S. Thurlow, *Biochem. J.*, **18**, 976 (1924).

nitrate reduction may be ascribed to the restriction of the supply of hydrogen from the native substrates, as for only amino purines. Therefore, a question arose in concern of the anticipation that the purine inhibition of the nitrate reduction is due to the retardation of the xanthine oxidase itself.

It has been often experienced that the ability to reduce nitrate is always accompanied with the xanthine and aldehyde oxidase activities in the course of purification. The activity of the dialysed homogenate of liver was extinguishingly decreased on account of the removal of inherent substrates. When 10^{-2}M acetaldehyde was supplemented, the activity to catalyse the reduction of nitrate and methylene blue was recovered. A similar reactivation was also attained by hypoxanthine and xanthine of 5×10^{-3}M. Howere, the decoloration of dye with hypoxanthine and xanthine was much faster than that with aldehyde, while nitrate reduction with the former two was lower than that of the later. According to the cattle liver[9], the homogenate was purified by repeated fractionation with ammonium sulfate and subsequent treatment on Ca phosphate gel. The enzyme preparation thus obtained was easily soluble in water and buffer making a clear pale yellow solution and its activity could not be assayed without use of substrates. The analogous experiment performed with the solution containing 30 mg preparations gave results that coincided well with those of the dialysed homogenate as indicated in Table II.

TABLE II. NITRATE REDUCTION AND METHYLENE BLUE DECOLORATION WITH DIALYSED HOMOGENATE AND PURIFLED PREPARATION

Enzyme	Dialysed homogenate			Purified preparation		
Substrate	acetaldehyde	hypoxanthine	xanthine	acetaldehyde	hypoxanthine	xanthine
NO_2 formed, μM	5.7	3.8	3.0	10.4	11.5	9.6
M.B. decol. time, min.	107	33	28	118	25	15

It may be infered, therefore, that the delivery of hydrogen to nitrate was partly obstructed by hypoxanthine and xanthine, by which the dehydrogenating velocity of enzyme had been highly activated, even if the xanthine oxidase could conjugate with the nitrate reduction. Hence, it is alternatively presumed that the nitrate reduction might be attributed to the catalytic action of the special enzyme which have very close properties with the xanthine oxidase, or that the active principle contributing to the nitrate reduction might differ from that of the xanthine oxidase action in the enzyme protein. In other words, hypoxanthine and xanthine influence inhibitory upon the enzyme or the active centre proper to nitrate reduction, while functioning as a substrate of the xanthine oxidase. In regard of the relation to the aldehyde oxidase, a similar assumption could be derived from the observation on the intracellular distribution of the activity[10]. In view of the structural analogy, the inhibition by amino purines and uric acid may be accounted for, at least to some extent, by the same mechanism, even if the decline of the nitrite formation could be attributed partly to competition against the dehydrogenase system. Thus, the principle catalysing the nitrate reduction could be permitted to be designate as the nitrate reductase.

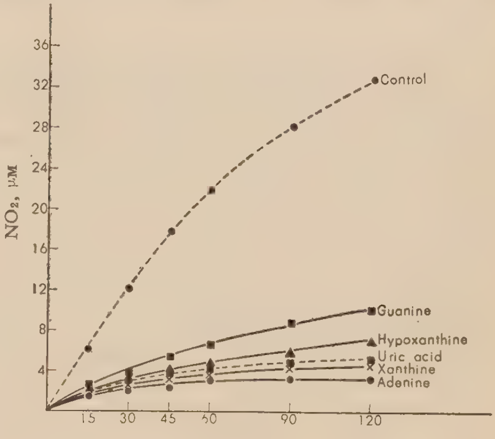

FIG. 1. Relation between the Nitrate Formation and Reduction time.

Since the degree of the inhibition varies with the enzymatic activity[6], the figures cited in Table I could not be compared with one another as they are, but imply the order of efficiency: adenine, xanthine, hypoxanthine and guanine.

It is also surmised that the inhibition may be indebted to uric acid but not to its precoursors, because the xanthine oxidase was working in the reaction mixture. However, the curves showing the relation between the nitrite formation and the reaction-time in Fig. 1 disclosed that the hindrance caused by purines approximately agreed with that already caused by uric acid in a short period of 15 min., so that this assumption may not be the case.

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[9] H. Omura and H. Takahashi, This Bulletin, to be published.

[10] H. Omura, to be published.

Effect of 6-Mercaptopurine. The purine skeleton consists of both pyrimidine and imidazole rings sharing two C atoms of 4 and 5 positions at the point of fusion. From one to all of the remaining C atoms of the purines examined above, were occupied with side chains of hydroxy and amino groups. A number of the modifications of the functional groups may lead to an antagonist. Recently such analogues have attracted widespread attention for medical purposes. 6-Mercaptopurine in which a sulfhydryl was introduced at the 6′C is one of such derivatives and is placed on the list of drugs as an antileucaemia having the commercial name of "Merun".

As shown in Table III, its inhibiting ability was established. Thus, it is the purine skeleton, not the side chains, that probably plays an important role in the inhibition. In supplemental experiments, it was found that histidine which contains the imidazole ring had no effect.

Effect of 8-Azapurines. There are many substituted compounds of purine of different kinds which also behave as the antagonist of the purine metabolism Now, 8-azaguanine and 8-azaxanthine, in which 8′C of the purine ring of guanine and xanthine is replaced by N, were tried. These substances are also employed for several purposes. Especially, the former had been put on market as an anti-cancer substance under the name of "Azan" and also antagonizes certain virus infections of plants[11]. Table IV affords a valid evidence that the ability to interfer with both reductions was maintained, and that, most effectively. However, the inhibition by azaguanine was mollified under the coexistence with 10^{-2}M acetaldehyde.

Then, uric acid, hypoxanthine and xanthine of 10^{-4} and 5×10^{-5}M were allowed to have an effect on the nitrate reducing system with 8-azaguanine of 5×10^{-5}M. We can see from Table V, that the inhibition by the cooperation of both purine and azaguanine was almost the same with that by the latter alone, regardless of

TABLE III. EFFECT OF 6-MERCAPTOPURINE ON THE NITRATE REDUCTION AND THE DECOLORATION OF METHYLENE BLUE

Conc. of purine added, M	0	10^{-3}	5×10^{-4}	10^{-4}	5×10^{-5}	10^{-5}
NO_2 formed, μM	40.0	8.0	14.9	29.8	38.4	40.8
Inhibition, %		80.0	62.7	25.5	4.0	0
M.B. decol. time, min.	14	25	—	21	14	14

TABLE IV. EFFECT OF 8-AZAPURINES ON THE NITRATE REDUCTION AND THE DECOLORATION OF METHYLENE BLUE

Conc. of purine added, M		0	10^{-3}	5×10^{-4}	10^{-4}	5×10^{-5}	10^{-5}
8-Azaguanine	NO_2 formed, μM	22.8	1.9	2.1	4.0	5.6	12.9
	Inhibition, %		91.6	90.8	82.5	75.4	43.4
	M.B. decol. time, min.	3	16	13	11	7	5
8-Azaguanine, Acetaldehyde	NO_2 formed, μM	22.1	5.1	5.5	5.8	9.0	14.6
	Inhibition, %		76.9	75.1	73.8	59.3	33.9
8-Azaxanthine	NO_2 formed, μM	19.1		1.2	1.8	3.3	7.4
	Inhibition, %			93.7	90.6	82.7	61.3
	M.B. decol. time, min.	7		13	13	—	7

TABLE V. COOPERATIVE EFFECT OF 8-AZAGUANINE AND SOME PURINES ON THE NITRATE REDUCTION

Purine added		None	Hypoxanthine		Xanthine		Uric acid	
Conc. of purine, M			10^{-4}	5×10^{-5}	10^{-4}	5×10^{-5}	10^{-4}	5×10^{-5}
NO_2 formed, μM	None	30.0	13.2	17.0	10.9	17.0	13.6	20.8
	Azaguanine	5.3	6.0	6.0	5.3	5.3	4.6	7.0

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[11] R.E.F. Matthews, *Nature*, **167**, 892 (1951).

TABLE VI. EFFECT OF N-METHYL PURINES ON THE NITRATE REDUCTION AND THE DECOLORATION OF METHYLENE BLUE

Conc. of purine added, M		0	10^{-2}	5×10^{-3}	10^{-3}	5×10^{-4}	10^{-4}
Caffeine	NO_2 formed, μM	10.8	10.5	10.5	10.5	10.7	10.9
	Inhibition, %		2.8	2.8	2.8	0.9	0
	M. B. decol. time, min.	37	37	37	37	37	37
Theophylline	NO_2 formed, μM	14.4	7.3	8.5	11.5	14.4	14.4
	Inhibition, %		49.3	40.3	20.0	0	0
	M. B. decol. time, min.	3	8	7	5.5	5	4

the amounts of the former two. So, it may be supposed that the effect of 8-azaguanine was predominant and obscured the effects of other purines, while it was weakened by aldehyde.

Effect of N-Methyl Purines. The purines studied so far have side chains at C atoms of the purine ring. On the contrary, N-methyl derivatives such as caffeine (1, 3, 7-trimethyl xanthine) and theophylline (1, 3-dimethyl xanthine) are contained in plant. As it is well-known, these compounds are also for medical use. It will be seen from the figures in Table VI that caffeine has almost no effect and theophylline more or less retarded the nitrate reduction and the decoloration of methylene blue, provided that a higher concentration of approximately ten-fold was applied.

Notwithstanding the kind of side chains at C atoms, the purines which possess remarkable inhibiting potency on the nitrate reduction have free-N at positions of 1, 3 and 7, having no relation to the replacement of 8′C by N, whereas N-methyl derivatives lost their agency. When one of such N had been freed, the inhibiting power was partially regained as in the case of theophylline. Therefore, it appears probable that one or all of N of purine may participate in the interference and that the variation of the groups at C atoms may not be so important but only control the degree of the inhibition. The other free N at 9, however, may have no part in the disturbance, because it is also included

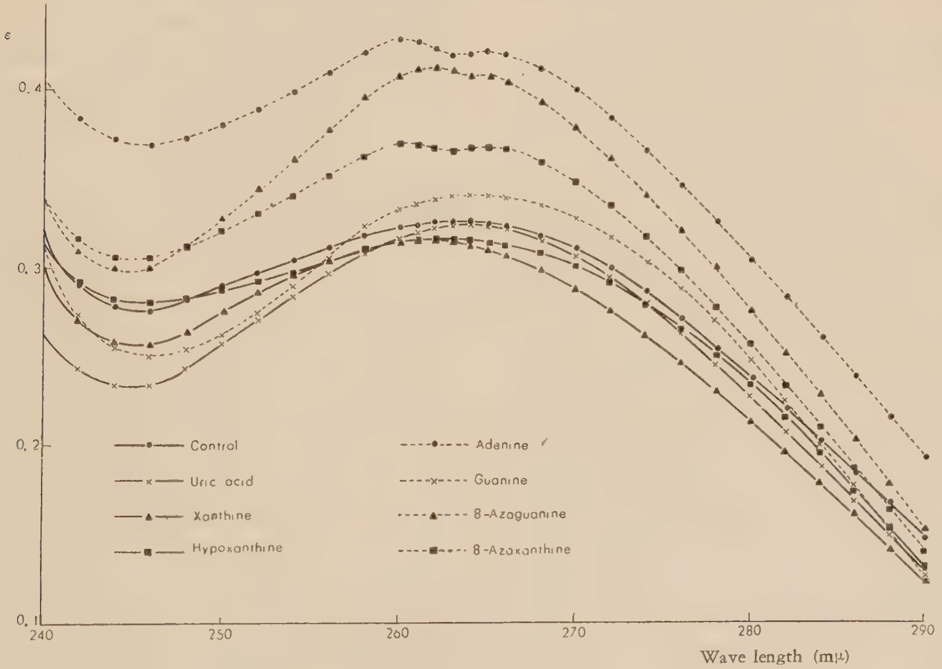

Fig. 2. Ultraviolet Spectra of the Mouse Liver Extract with Some Purines.

in caffeine and theophylline. As for the microbial nitrate reductase[12,13], it was established that FAD and TPN play an essential role in the transfer of hydrogen between original donors and nitrate bringing about the nitrate reduction. Also, on the liver, some suggestions were obtained that flavine compound[14] and DPN[15], another important coenzyme in the hydrogen transport systems in nature, may behave as a cofactor. In a molecule of these nucleotides, adenine is included by combining with sugar through 9′N leaving N of 1, 3 and 7 free. Therefore, it is presumed that one or more free Ns of adenine moiety in the coenzyme may combine with the enzyme protein and that hindrance by purine may be attributed to the competition with adenine of the cofactors. Hence, the highest inhibition by adenine and non-participation of 9′N may reasonably be explained.

This assumption might be supported in part by Fig. 2 showing variation of ultraviolet absorption spectrum which suggest the combination of purines with some protein. The activity of the nitrate reduction was found to be most prosperous in the cytoplasmic fluid of the liver cell. Ten % (w/v) homogenate prepared as above was centrifuged further at a higher speed of 15000 r.p.m. for 60 min. and the clear supernatant was obtained. The ultraviolet spectrum was measured with a Beckman spectrophotometer after being diluted 40-fold and, a maximum at 264 mμ and a minimum at 246 mμ were checked. Then, on the mixture of 2 ml of liver extract, 0.4 ml of 5×10^{-3}M purine and 1.6 ml of water, the spectra were estimated after a 20-times dilution. Inspection of the extinction curves revealed that there were obvious variation in the region around the maximum corresponding to the protein, especially, the increment of the absorptions and the shift of the peak, were provoked by adenine, guanine, azaguanine and azaxanthine. However, little alteration was given rise by hypoxanthine, xanthine and uric acid. These changes may imply the possibility of combination of purine with certain protein. The most excellent variations by adenine and azapurines may show this conjecture in connection with the degree of the inhibitions by purines.

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[12] F. Egami and R. Sato, *J. Chem. Soc. Japan*, **69**, 160 (1948).

[13] A. Nason and H. J. Evans, *J. Biol. Chem.*, **202**, 655 (1953).

[14] H. Omura, *Sci. Bull. Fac. Agr. Kyushu Univ.*, **16**, 225 (1957).

[15] H. Omura, unpublished.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 6, p. 393~398, 1958]

Relationship between Stereoisomerism and Biological Activity of Pyrethroids

Part III. The Toxicity of Allethronyl Homochrysanthemates and the Related Compounds

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Higher homologous acids of chrysanthemic acid described in the previous papers were esterified with (±)-allethrolone. The toxicity of these esters and the related compounds were evaluated by the topical application method to *Musca domestica vicina* Macq. The allethronyl homochrysanthemate (entry Nos. 4, 5) was shown to be toxic and the dextrorotatory form was far more toxic than the laevorotatory one. Further elongation of the ester linkage resulted in a loss of toxicity. The *cyclo*butane carboxylic acid ester (entry No. 10) was shown to be toxic and so, the *cyclo*propane ring might be replaced to some extent by the *cyclo*butane ring, provided the other requirements were fulfiled. However, further elongation of the ester linkage also reduced the toxicity. The lactones (entry Nos. 12–16) obtained by the hot sulfuric acid treatment were non-toxic.

In order to obtain some informations concerning the relationship between stereochemical configuration and toxicity of pyrethroids, the authors intended to lengthen the ester linkage connecting the *cyclo*propane acid moiety with the *cyclo*pentenolone moiety. At first, a carbon chain extention in part of chrysanthemic acid was attempted for this purpose.

Some higher homologues of chrysanthemic acid have been prepared by the Arndt-Eistert synthesis and the structures thereof together with those of the related compounds have also been demonstrated in previous papers[1]. These homologous acids were converted by the action of thionylchloride into the corresponding acid chlorides and then, esterified with (±)-allethrolone according to the general procedure described by La Forge[2]. The esters thus obtained, were purified by displacement chromatography through a column of activated alumina and then the purity was estimated by means of polarography.

Prior to this, precise polarographical studies had shown that these higher homologues of allethrin behaved polarographically in exactly the same manner as allethrin did, and the quantitative determination using the calibration curve of α-*dl*-*trans*-allethrin as a standard and by employing a molecular weight factor was quite sufficient.

The toxicity of these esters and the related compounds, together with that of α-*dl*-*trans*-allethrin as a standard of reference were made by the topical application method using the common housefly, *Musca domestica vicina* Macquart. The median knock down concentrations after 30 minutes and median lethal concentrations after 24 hours, determined by Bliss' probit transformation method, are given in Table I.

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* Institute for Chemical Research, Kyoto University.

[1] Y. Katsuda, T. Chikamoto and Y. Inouye, This Bulletin, **22**, 185 (1958); Y. Katsuda and T. Chikamoto, This Bulletin, **22**, 330 (1958).

[2] F. B. La Forge and W. E. Barthel, *J. Org. Chem.*, **12**, 199 (1947).

TABLE I. EVALUATION OF RELATIVE EFFECTIVENESS ON TOXICITY OF ALLETHRONYL ESTERS OF HIGHER HOMOLOGOUS ACIDS AND THE RELATED COMPOUNDS AS COMPARED WITH α-*dl*-*trans*-ALLETHRIN

Series	Entry	Material	Median Knockdown concentration		Median Lethal concentration	
			KC-50 %	Relative Knockdown effectiveness	LC-50 %	Relative Lethal effectivness
1		α-*dl*-*trans*-allethrin	0.075	1.00	0.127	1.00
	9	*dl*-allethronyl *dl*-*cis*-chrysanthemate	0.035	2.15	0.069	1.85
2		α-*dl*-*trans*-allethrin	0.078	1.00	0.195	1.00
	10	*dl*-allethronyl *dl*-*cis*-3-*iso*butenyl-2, 2-dimethyl-*cyclo*butane-1-carboxylate	0.748	0.11	0.357	0.55
3		α-*dl*-*trans*-allethrin	0.193	1.00	0.312	1.00
	4	*dl*-allethronyl *d*-*trans*-homochrysanthemate	0.551	0.35	0.422	0.74
4		α-*dl*-*trans*-allethrin	0.077	1.00	0.133	1.00
	2	*dl*-allethronyl-*d*-*trans*-chrysanthemate	0.006	13.50	0.027	4.88
5		α-*dl*-*trans*-allethrin	0.102	1.00	0.165	1.00
	3	*dl*-allethronyl *l*-*trans*-chrysanthemate	0.289	0.35	0.467	0.35
6		α-*dl*-*trans*-allethrin	0.090	1.00	0.158	1.00
	1	*dl*-allethronyl *dl*-*trans*-chrysanthemate	0.014	6.56	0.039	4.09
	5	*dl*-allethronyl-*l*-*trans*-homochrysanthemate	(240.200)*	0.0004	(90.146)*	0.0018
7		α-*dl*-*trans*-allethrin	0.117	—	0.191	—
	7	*dl*-allethronyl-*d*-*trans*-β-(3-*iso*butenyl-2, 2-dimethyl*cyclo*propane-1)-propionate	—	—	—	—
	8	*dl*-allethronyl-*l*-*trans*-β-(3-*iso*butenyl-2, 2-dimethyl*cyclo*propane-1)-propionate	—	—	—	—
	6	*dl*-allethronyl *dl*-*trans*-β-(3-*iso*butenyl-2, 2-dimethyl*cyclo*propane-1)-propionate	—	—	—	—
	11	*dl*-allethronyl *dl*-*cis*-3-*iso*butenyl-2, 2-dimethy*cyclo*butane-1-acetate	—	—	—	—
	12	($\pm$)-δ, δ-dimethyl-γ-*iso*butenyl-δ-valerolactone	—	—	—	—
	13	(+)-δ, δ-dimethyl-γ-*iso*butenyl-δ-valerolactone	—	—	—	—
	14	($\pm$)-γ-(1′, 1′, 4′-trimethyl-pent-2′-enyl)-butyrolactone	—	—	—	—
	15	γ-lactone (from ($\pm$)-*cis*-3-*iso*butenyl-2, 2-dimethyl*cyclo*butane-1-acetic acid)	—	—	—	—
	16	($\pm$)-pyrocin	—	—	—	—

* The value in parentheses is not realized

The allethronyl homochrysanthemate (entry Nos. 4, 5) with a longer ester linkage by one methylene, than the parent chrysanthemate was shown to be toxic, but further elongation of the linkage (entry Nos. 6–8) caused the complete loss of toxicity. Also, in the homochrysanthemate, the dextrorotatory form (entry No. 4) was far more toxic than the laevorotatory one (entry No. 5) as was the case in the chrysanthemates, but the *d*/*l* ratio was not improved by such molecular change in contrast to our expectation. The implication is as follows: it is the absolute configuration at the asymmetric $C_{(1)}$ atom that is important for insecticidal activity, and the molecular conformation of the optically isomeric chrysanthemates is not improved by such molecular modification in ester linkage towards the form more appropriate for the "three points contact".

It is noteworthy that the *cyclo*butanecarboxylate (entry No. 10) shows an insecticidal toxicity, although it is lower. As the structural requirements essential for the high

TABLE II. REFRACTION INDICES AND HALFWAVE POTENTIAL OF SYNTHETIC PYRETHROIDS

Entry	Material	n_D^{20}	Half-wave potential v.s. S.C.E.
1.	*dl*-allethronyl-*dl*-*trans*-chrysanthemate	1.5060	−1.24
2.	-*d*-*trans*-chrysanthemate	1.5072	–
3.	-*l*-*trans*-chrysanthemate	1.5076	—
4.	-*d*-*trans*-homochrysanthemate	1.5019	−1.23
5.	-*l*-*trans*-homochrysanthemate	1.5021	—
6.	-*dl*-*trans*-β-(3-*iso*butenyl-2, 2-dimetyl*cyclo*propane-1)-propionate	1.5008	−1.24
7.	-*d*-*trans*- ,, ,,	1.5010	—
8.	-*l*-*trans*- ,, ,,	1.5022	—
9.	-*dl*-*cis*-chrysanthemate	1.5090	−1.24
10.	-*dl*-*cis*-3-*iso*butenyl-2, 2-dimethyl*cyclo*butane-1-carboxylate	1.4960	−1.25
11.	-*dl*-*cis*-3-*iso*butenyl-2, 2-dimethyl*cyclo*butane-1-acetate	1.5008	−1.25

toxicity of rethronyl chrysanthemates, the unsaturated *iso*butenyl side chain, gem.-dimethyl groups and the *cyclo*propane ring in the acidic part of the molecule have been pronounced by many workers. Now it seems that the *cyclo*propane ring might be replaced to some extent by the *cyclo*butane ring so far as other requirements are fulfiled. However, further elongation of the ester linkage erduced the toxicity to that inmeasurable, (entry No. 11), as was the case with the *trans*-homochrysanthemates (entry No. 6). The lactones (entry Nos. 12–16) were non-toxic so for caution's sake, pyrocin was reexamined for reference. The bioassay results by means of topical application showned that pyrocin was non-toxic against the common house flies in agreement with Harper's conclusion[3] in spite of Matsui's report[4] of high toxicity for this lactone.

EXPERIMENTAL

General procedure. *dl*-Allethronyl *dl*-*trans*-homochrysanthemate.

Synthetic *dl*-allethrolone, n_D^{25} 1.5135 (2.3 g., 0.015 mole) and 1.7 ml. of dry pyridine in 15 ml. of dry benzene were added to *dl*-*trans*-homochrysanthemoyl chloride (3.2 g., 0.016 mole) synthesised, by the authors previously[1], in 20 ml. of benzene.

Pyridine hydrochloride separated in a short time, and the reaction mixture was allowed to stand for about 24 hours at room temperature. The crystalline material was filtered off, and the solution was washed with water, sodium bicarbonate solution, dilute hydrochloric acid and water. After removal of the solvent *in vacuo* in nitrogen atmosphere, the residue was dissolved in petroleum ether. Then the purification was undertaken by passing through an alumina column. After removal of the solvent *in vacuo* in nitrogen atmosphere, the purity was determined by the polarographic method, in which α-*dl*-*trans*-allethrin was used as a standard substance.

By the same procedures, allethrolone esters of several chrysanthemic acid homologues were prepared and their refraction indices and half-wave potential are shown in Table II.

Biological assay

Materials: Sixteen compounds as listed Table I and α-*dl*-*trans*-allethrin standards of comparison were prepared for tests by dissolving in acetone at six to eight selected concentrations so as to cause responses low to high.

Procedure: The tests were carried out by the topical application method on adults the common house fly, *Musca domestica vicina* Macq., reared by the standard procedure using a culture medium prepared with "okara"[5]. While the adult flies were immolbilized by a low temperature, 0.0005 cc of a toxicant solution of known-concentration in acetone was applied topically to the scutum portion of each fly with a micrometer-operated syringe. Though no determination of the sex

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[3] L. Crombie, S. H. Harper and R. A. Thompson J, *Sci. Food Agric.* **2**, 427 (1951).

[4] M. Matsui, *Botyu-Kagaku*, **15**, 18 (1950)

[5] S. Nagasawa, *Bull. Inst. Chem. Research, Kyoto Univ.*, **34**, 101 (1956).

TABLE III. CONCENTRATION-KNOCKDOWN AND CONCENTRATION-MORTALITY OF ADULTS OF THE COMMON HOUSE FLY, *Musca domestica vicina* Macq. FOR *α-dl-trans* ALLETHRIN AND ALLETHRONYL ESTERS OF HIGHER HOMOLOGOUS ACIDS AND THE RELATED COMPOUNDS APPLIED TOPICALLY AS ACETONE SOLUTION

Series	Entry	Material	Concentration	Number of insects	Knockdown in 30 minutes	Mortality in 1 day
1		*α-dl-trans*-allethrin	1/1 mg/ml	74	78.4%	97.3%
			1/2	68	76.5	95.6
			1/4	79	79.8	79.8
			1/8	87	75.9	49.4
			1/16	70	40.0	24.3
			1/32	81	12.4	2.5
	9	*dl*-allethronyl *dl-cis*-chrysanthemate	1/1	88	97.7	100.0
			1/2	80	91.3	100.0
			1/4	74	82.4	93.2
			1/8	75	70.7	69.3
			1/16	66	66.7	43.9
			1/32	73	48.0	23.3
2		*α-dl-trans*-allethrin	1/1	64	90.8	95.3
			1/2	73	86.3	87.7
			1/4	73	63.0	58.9
			1/8	79	59.4	27.9
			1/16	82	52.4	13.4
			1/32	79	29.1	2.5
	10	*dl*-allethronyl-*dl-cis*-3-*iso*butenyl-2, 2-dimethyl-*cyclo*butane-1-carboxylate	1/1	96	61.5	94.8
			1/2	99	35.4	73.7
			1/4	84	20.2	26.2
			1/8	82	15.9	15.9
			1/16	73	1.4	5.5
			1/32	72	2.8	2.8
3		*α-dl-trans*-allethrin	1/1	94	91.5	85.1
			1/2	72	79.2	66.7
			1/4	82	50.0	46.3
			1/8	83	43.3	19.3
			1/16	72	13.9	5.6
			1/32	73	9.6	0.0
	4	*dl*-allethronyl *d-trans*-homochrysanthemate	1/1	67	52.2	79.1
			1/2	70	51.4	55.7
			1/4	74	43.2	37.8
			1/8	80	35.0	10.0
			1/16	73	23.3	5.5
			1/32	72	16.7	1.4
4		*α-dl-trans*-allethrin	1/1	81	96.3	98.8
			1/2	79	93.7	94.9
			1/4	72	88.9	86.1
			1/8	88	58.0	39.8
			1/16	62	54.8	16.1
			1/32	72	18.1	5.6
	2	*dl*-allethronyl-*d-trans*-chrysanthemate	1/8	79	100.0	98.7
			1/16	79	98.7	74.7
			1/32	92	95.7	63.0
			1/64	77	89.6	23.4
			1/128	88	71.6	10.2
			1/256	74	27.0	2.7
			1/512	77	7.8	0.0
5		*α-dl-trans*-allethrin	1/1	78	97.4	100.0

TABLE III

Series	Entry	Material	Concentration	Number of insects	Knockdown in 30 minutes	Mortality in 1 dfy
			1/2 mg/ml	91	91.2%	87.9%
			1/4	80	75.0	67.5
			1/8	80	48.8	36.3
			1/16	92	43.5	16.3
			1/32	96	13.5	0.0
	3	*dl*-allethronyl-*l*-*trans*-chrysanthemate	1/1	75	64.0	72.0
			1/2	73	60.3	53.4
			1/4	69	47.8	34.8
			1/8	75	34.7	8.0
			1/16	74	31.1	8.1
			1/32	83	28.9	2.4
6		α-*dl*-*trans*-allethrin	1/1	59	98.3	68.3
			1/2	65	92.3	87.7
			1/4	53	83.0	66.0
			1/8	49	61.2	38.8
			1/16	58	43.1	19.0
			1/32	66	13.6	0.0
	1	*dl*-allethronyl-*dl*-*trans*-chrysanthemate	1/16	59	91.5	69.5
			1/32	53	83.0	39.6
			1/64	60	55.0	18.3
			1/128	56	33.9	14.3
			1/256	59	5.1	3.4
			1/512	56	0.0	0.0
	5	*dl*-allethronyl-*l*-*trans*-homochrysanthemate	16/1	58	36.2	72.4
			8/1	56	16.1	48.2
			4/1	69	5.8	17.4
			2/1	66	0.0	4.6
			1/1	75	0.0	0.0
7		α-*dl*-*trans*-allethrin	1/1	95	91.6	95.8
			1/2	78	75.6	76.9
			1/4	64	78.1	62.5
			1/8	68	35.3	14.7
			1/16	70	41.4	14.3
			1/32	71	24.0	5.8
	7	*dl*-allethronyl-*d*-*trans*-β-(3-*iso*butenyl-2, 2-dimethyl*cyclo*propane-1)-propionate	1/1	103	0.0	0.0
			1/2	90	0.0	0.0
			1/4	78	0.0	0.0
			1/8	80	0.0	0.0
			1/16	81	0.0	0.0
			1/32	76	0.0	0.0
	8	*dl*-allethronyl-*l*-*trans*-β-(3-*iso*butenyl-2, 2-dimethyl*cyclo*propane-1)-propionate	1/1	128	0.0	0.0
			1/2	84	0.0	0.0
			1/4	109	0.0	0.0
			1/8	91	0.0	0.0
			1/16	88	0.0	0.0
			1/32	73	0.0	0.0
	6	*dl*-allethronyl-*dl*-*trans*-β-(-3-*iso*butenyl-2, 2-dimethl*cyclo*propane-1)-propionate	1/1	73	0.0	0.0
			1/2	78	0.0	0.0
			1/4	81	0.0	0.0
			1/8	71	0.0	0.0
			1/16	77	0.0	0.0
			1/32	71	0.0	0.0

Table III

Series	Entry	Material	Concentration	Number of insects	Knockdown in 30 minutes	Mortality in 1 day
	11	*dl*-allethronyl-*dl*-*cis*-3-*iso*butenyl-2, 2-dimethyl*cyclo*butane-1-acetate	1/1 mg/ml	86	0.0%	0.0%
			1/2	89	0.0	0.0
			1/4	70	0.0	0.0
			1/8	74	0.0	0.0
			1/16	63	0.0	0.0
			1/32	71	0.0	0.0
	12	(±)-δ, δ-dimethyl-γ-*iso*butenyl-δ-valerolactone	1/1	67	0.0	0.0
	13	(+)-δ, δ-dimethyl-γ-*iso*butenyl-δ-valerolactone	1/1	71	0.0	0.0
	14	(±)-γ-(1′, 1′, 4′-trimethyl-pent-2′-enyl)-butyrolactone	1/1	69	0.0	0.0
	15	γ-lactone (from (±)-*cis*-3-*iso*butenyl-2, 2-dimethyl*cyclo*butane-1-acetic acid)	1/1	74	0.0	0.0
	16	(±)-pyrocin	1/1	85	0.0	0.0

was made in advance of the tests, determination results of the tests revealed that the sex ratio was 1 : 1 in almost all cases. After treatment, the flies were transfered to clean petri-dishes, provided with wheat paste as a diet, and kept at room temperature until knockdown counts, 30 minutes later and mortality counts 24 hours later were made.

Results: The knockdown and mortality data are summarized in Table III, where they are grouped according to the different populations of flies (series 1, 2, etc.). Median knockdown and lethal concentrations were assessed from the equations of concentration-knockdown and concentration-mortality regression lines, and relative toxicities were calculated as inverse ratios of KC-50's and LC-50's, as given in Table I.

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Electrolytic Reductions of Diphosphopyridine Nucleotide, Triphosphopyridine Nucleotide, and Cytochrome c at Controlled Potentials*

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It has been reported by several persons, including one of the authors, that DPNH obtained by the electrolytic reduction at controlled potentials is not fully active in respect to the reaction with alcohol dehydrogenase system. But statements concening the percentage of activity and the conditions which affect the activity were not identical. This article deals with these problems by reducing DPN electrolytically under different conditions followed by enzymatic examinations. The electrolytic reductions of TPN and cytochrome c were also performed. Though the data presented are rather complicated, the most active DPNH was prepared in tripolyphosphate buffer with platinum electrode at -2.0 volt vs. S.C.E., under ice-cooling. The effects of phosphates and electrolytic potential are discussed.

I. INTRODUCTION

In the previous article[1], it was reported that reduced diphosphopyridine nucleotide (DPNH), which was partially active in respect to the alcohol dehydrogenase system, was obtained by electrolytic reduction of diphosphopyridine nucleotide (DPN). Essentially same results were also obtained by B. Ke[2~4], M.B. Yarmolinsky & S.P. Colowick[5], and R.F. Powning & C.C. Kratzing[6] in independent experiments.

Their observations, however, were not completely identical in several respects. Yarmolinsky & Colowick and one of the authors obtained about 30% or more active DPNH electrolytically by using mercury cathode in phosphate buffer solution, while Ke obtained completely inactive DPNH with mercury cathode in tris(hydroxymethyl)aminomethane (tris) buffer solution. But later he obtained 37% and 56% active DPNH using platinum and lead cathode, respectively. On the other hand, Powning and Kratzing reported that the activity was dependent upon the buffer solution, i.e., the activities of DPNH obtained in tris-, phosphate-, and pyrophosphate buffer solutions were 3, 55, and 50–80%, respectively. They also reported that the activity of electrolytically reduced triphosphopyridine nucleotide (TPNH) was amounted to 94%.

We attempted to reduce DPN and triphosphopyridine nucleotide (TPN) under different conditions so as to examine these different observations. We were particularly interested in the following three points. The first point was the function of phosphate and pyrophosphate indicated in the article of Powning & Kratzing. The problem arising was that if phosphate functions in producing 30–50% active DPNH, and pyrophosphate functions in producing 50–80% active DPNH, how much activity would the product have, when DPN is reduced in the presence of tripolyphosphate or polyphosphate.

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* Electrolytic Reduction of Physiologically Important Substances at Controlled Potentials. Part II.

1) T. Kôno, *Bull. Agr. Chem. Soc. Japan*, **21**, 115 (1957).
2) B. Ke, *Arch. Biochem. Biophys.*, **60**, 505 (1956).
3) B. Ke, *Biochim. Biophys. Acta*, **20**, 547 (1956).
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5) M. B. Yarmolinsky and S. P. Colowick, *Biochim. Biophys. Acta*, **20**, 547 (1956).
6) R. F. Powning and C. C. Kratzing, *Arch. Biochem. Biophys.*, **66**, 249 (1957).

The second point was the effect of the cathode. If platinum electrode is more effective than mercury to increase the enzymatic activity of DPNH, and if pyrophosphate is much more effective than tris buffer, then, how much activity could be expected when DPN is reduced in pyrophosphate buffer using platinum as the cathode.

The third problem, in which we were interested, was the reduction of cytochrome c, because it seemed to us that the electrolytic reduction would produce nothing else but a fully active reduced cytochrome c.

Experiments pertaining to these questions are presented in this article.

II. MATERIALS AND REAGENTS

DPN and TPN: DPN and TPN were purchased from the Nutritional Biochemical Co., U.S.A.

Alcohol dehydrogenase: Alcohol dehydrogenase was prepared according to the method of Racker[7].

TPNH-glutathione reductase: TPNH-glutathione reductase was prepared according to Kaplan, Colowick, and Wenfeld[8].

Glutathione: Oxidized form of glutathione was prepared according to Mapson and Goddard[9].

Cytochrome c: Cytochrome c was prepared according to Hagiwara[10].

Cytochrome a_3: Cytochrome a_3 was prepared according to Keilin and Hartree[11].

Other reagents: Reagents of c. p. grade were used in the experiments.

The polyphosphate used was the so called Metaphosphate purchased from Koso Chemical Co., Tokyo.

III. METHODS

1. Reduction of DPN, TPN, and cytochrome c. Electrolytic reduction of DPN, TPN, and cytochrome c were performed as described in the previous paper[1]. Fine platinum wool was used as the cathode in some of the experiments. Cathode potentials were expressed versus saturated calomel electrode (S.C.E.). The pH of the medium was adjusted to the starting value after reduction.

2. The determination of enzymatic activity of DPN. DPNH was incubated with acetaldehyde and alcohol dehydrogenase for fifteen minutes, and the optical density (O.D.) at 340 mμ was determined with a Hitachi spectrophotometer. The optical densities of DPN and DPNH at 340 mμ were also determined in the mixture which did not contain alcohol dehydrogenase. The composition of the reaction mixture was as follows:

DPNH	0.15 μ mole
Acetaldehyde	100 μ moles
Alcohol dehydrogenase	*
Buffer solution	3.0 ml

* Alcohol dehydrogenase was diluted so that 0.2 μ mole of DPNH in 3 ml buffer solution was completely oxidized in 5–10 min.

The enzymatic activity of DPNH was calculated from the following equation:

$$\frac{\text{O.D. of DPNH} - \text{O.D. after oxidation}}{\text{O.D. of DPNH} - \text{O.D. of DPN}} \times 100 = \text{Activity\%}$$

3. Determination of enzymatic activity of TPNH. The enzymatic activity of TPNH was determined in the same way as in the case of the determination of DPNH, using 0.15 μ mole of TPNH, 2 μ moles of oxidized form of glutathione, and an adequate amount of TPNH-glutathione reductase.

4. Determination of enzymatic activity of reduced cytochrome c. An adequately diluted 0.1 ml solution of cytochrome a_3 was added to 4 ml of 10^{-5}M reduced cytochrome c in a buffer solution. The mixture was left at room temperature for thirty minutes, and centrifuged. The optical density of the supernatant at 552 mμ was determined spectrophotometrically. The enzymatic activity of the electrolytically reduced cytochrome c was determined from the following equation, because a partially reduced cytochrome c was used as the material.

$$\text{Activity\%} = \frac{(a-c)-(b-d)^{*}}{(a-b)}$$

* Optical density at 552 mμ of the solution containing the same moles of:

a: electrolytically reduced cytochrome c.

b: starting material, 0.089 (cf. Table V).

c: electrolytically reduced cytochrome c after being oxidized by cytochrome a_3.

d: starting material after being oxidized by cytochrome a_3, 0.073 (cf. Table V).

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[11] D. Keilin and E. F. Hartree, *Proc. Roy. Soc.* (*London*), B**129**, 277 (1940).

IV. EXPERIMENTS AND RESULTS

A. Electrolytic reduction of DPN. By the use of DPNH, which was obtained by reducing DPN with sodium dithionite, the rate of the oxidation of DPNH by alcohol dehydrogenase system was measured. The buffer was a 0.1 M solution of sodium pyrophosphate, pH 7.0. The results are shown in Table I.

TABLE I. THE RATE OF OXIDATION OF DPNH BY ALCOHOL DEHYDROGENASE SYSTEM

Time, mins.	Optical density at 340 $m\mu$.
0 (DPNH)	0.213
3	0.054
5	0.050
7	0.050
15	0.050
28	0.050
DPN solution	0.049

The enzymatic activities of DPNH obtained under different conditions are summarized in Table II.

B. Electrolytic reduction of TPN. Using TPN, which was reduced by sodium dithionite, the rate of the oxidation of TPNH by TPNH-glutathione reductase was measured. The buffer was a 0.1 M solution of sodium pyrophosphate, pH 7.0. The results are shown in Table III.

TABLE III. THE RATE OF OXIDATION OF TPNH BY TPNH-GLUTATHIONE REDUCTASE SYSTEM

Time, min.	Optical density at 340 $m\mu$
0 (TPNH)	0.240
5	0.086
10	0.065
15	0.045
20	0.045
TPN	0.044

Enzymatic activities of TPNH obtained electrolytically under different conditions are summarized in Table IV.

TABLE II. ENZYMATIC ACTIVITIES OF DPNH OBTAINED UNDER DIFFERENT CONDITIONS

Cathode	Buffer	Concn. M	pH	Volt	Time mins.	Activity %	Average activity
Mercury	sodium pyrophosphate	0.10	7.0	−1.6	45	20.8	
		0.10	7.0	−1.6	45	17.5	
		0.10	7.0	−1.75	45	35.7	
		0.10	7.0	−1.9	30	43.3	
		0.05	7.0	−2.0	15	41.6	50.2
		0.10	7.0	−2.0	15	37.1	
		0.05	9.0	−2.0	15	47.6	
		0.10	9.0	−2.0	15	55.8	
		0.07	9.2	−2.0	45	44.7	
		0.10	9.2	−2.0	45	62.0	
		0.05	9.0	−2.0	15	62.9	
	sodium tripolyphosphate	0.025	9.0	−2.0	15	42.4	51.7
		0.05	9.0	−2.0	15	60.0	
		0.10	9.0	−2.0	15	52.3	
		0.05	7.0	−2.0	15	52.2	
		0.10	7.0	−2.0	15	(10.0?)	
	sodium polyphosphate	0.025	9.0	−2.0	15	21.0	30.4
		0.05	9.0	−2.0	15	25.0	
		0.10	9.0	−2.0	15	45.2	
	tris.	0.10	7.5	−1.8	30	19.5	19.9
		0.10	7.5	−2.0	30	20.3	
Platinum	sodium pyrophosphate	0.05	9.0	−2.0	20	58.2	54.5
		0.10	9.0	−2.0	20	66.1	
		0.05	7.0	−2.0	20	39.9	
		0.10	7.0	−2.0	20	59.0	
	sodium tripolyphosphate	0.05	7.0	−2.0	20	65.5	72.4
		0.10	7.0	−2.0	20	72.6	
		0.10	7.0	−2.0	30	72.2	
		0.10	7.0	−2.0	40	75.8*	
		0.10	7.0	−3.0	20	75.8*	

* reduced at 0~5°C.

TABLE IV. ENZYMATIC ACTIVITIES OF DPNH OBTAINED UNDER DIFFERENT CONDITIONS

Cathode	Buffer	Concn., M	pH	Volt	Time, min.	Activity %*
Platinum	Sodium pyrophosphate	0.1	7.0	−1.8	30	95
		0.1	7.0	−3.0	30	79

* The activity is expressed by Δoptical density×1000/2 min.

C. Electrolytic reduction of cytochrome c.

1. The rate of reduction. The rates of the electrolytic reduction of cytochrome c in 0.1 M sodium tripolyphosphate solution at different cathode potentials are illustrated in Fig. 1, in which the degree of the reduction is expressed by the optical density of the solution at 552 mμ. Only platinum was used as the cathode, because when mercury was tried, it was divided into small drops which were insulated from one another, in the presence of protein.

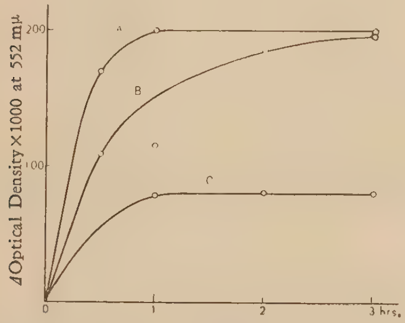

FIG. 1. Rates of the Reduction of Cytochrome c.
A: at −1.1, B: at −0.9, and C: at −0.6 volt, vs. S.C E.

As it was found from Fig. 1, that reduction took place far below its normal potential, the relationship between the rate of reduction and the amount of electrolytic current, which was the measure of hydrogen formation was examined. The results are illustrated in Fig. 2, where the degree of the reduction is expressed by optical density of the solution after being reduced for one hour in 0.1 M sodium triphosphate buffer solution at pH 7.0.

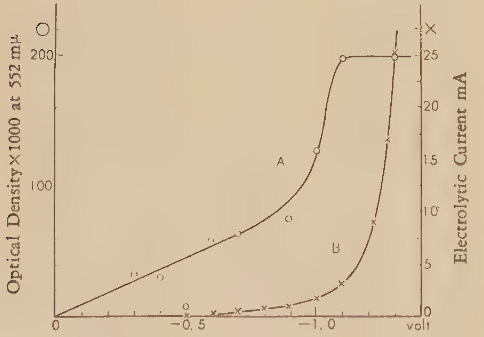

FIG. 2. The Degree of the Reduction of Cytochrome c and the Amount of the Electrolytic Current under Different Cathode Potentials.
A: The degree of the reduction after 1 hr. B: The amount of the electrolytic current.

The absorption spectrum of the reduction products are illustrated in Fig. 3, together with the optical density of the oxidized cytochrome c at 552 mμ.

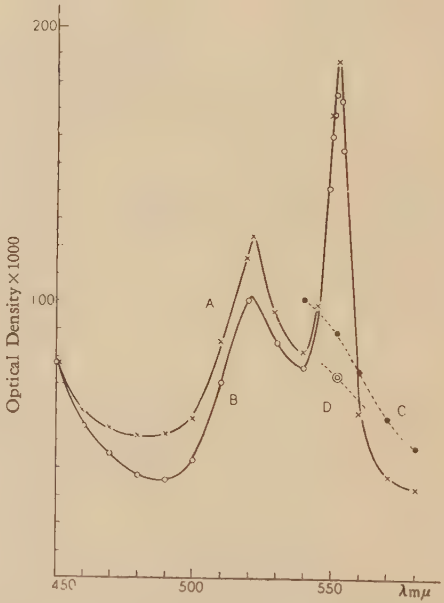

FIG. 3. Absorption Spectra of Cytochrome c.
A: ×——× reduced electrolytically, B: ○——○ reduced with sodium dithionite, C: ●——● starting material, D: ◉ C was oxidized with cytochrome a_3

2. The rate of oxidation. Using cytochrome c, which was reduced with sodium dithionite, the rate of decrease of the optical density at 552 mμ was measured by means of cytochrome a_3. The results are shown in Table V. The buffer was 0.1 M sodium tripolyphosphate.

TABLE V. THE RATE OF THE OXIDATION OF CYTOCHROME c BY MEANS OF CYTOCHROME a_3

Time, min	Optical density at 552 mμ
0 (reduced form)	0.180
3	0.125
6	0.112
9	0.104
12	0.097
15	0.091
18	0.087
21	0.082
24	0.075
27	0.075
30	0.075
starting material	0.089
oxidized form*	0.073

* The starting material was oxidized with cytochrome a_3.

3. The enzymatic activity. The enzymatic activities of electrolytically reduced cytochrome c are shown in Table VI.

TABLE VI. ENZYMATIC ACTIVITIES OF ELECTROLYTICALLY OBTAINED CYTOCHROME c

Cathode voltage	Time min.	Concn. of the buffer*	Activity %
−1.0	50	0.05	64.7
−1.1	50	0.05	61.0
−1.1	30	0.10	58.8

* Sodium tripolyphosphate, pH 7.0 was used.

V. DISCUSSION AND CONCLUSION

A. On the reduction of DPN. Among several factors, there were at least three factors which increased the enzymatic activity of DPNH. The effect of phosphate and pyrophosphate had already been recognized[6]. But we found that although tripolyphosphate was as effective as pyrophosphate, polyphosphate was less effective than those two. Its effect was comparable with that of orthophosphate.

The difference of mercury and platinum as the electrode was insignificant in pyrophosphate solution, but was significant in tripolyphosphate solution. The most active DPNH, 76%, was obtained with platinum cathode in tripolyphosphate under cooling.

Thirdly, the rate of the reduction of DPN in the vicinity of its normal potential is very slow, as was already reported by several authors, and the enzymatic activity of the product, DPNH, was higher when more negative potential was applied. The effect of a more negative potential might be due to the effect of hydrogen, because water was hydrolyzed at −2.0 volt v.s. S.C.E., and we wold rather prefer to consider that the enzymatically active DPNH might be formed by the hydrogen which was produced electrolytically.

B. On the reduction of TPN. TPNH having 95% enzymatic activity was obtained as reported by Powning and Kratzing[6]. As the experimental conditions were identical with those which have been applied in the reduction of DPN, the difference may be attributed to the additional phosphate in TPN. The interaction of phosphate with DPN or TPN is not clear at the present stage, but it seems that phosphate or its polymer would activate the third cardon of pyridine ring. In fact, DPNH dissolved in phosphate buffer is autoxidizable, but this is not the case in tris buffer.

C. On the reduction of Cytochrome c. We had expected that cytochrome c would be reduced in the vicinity of its normal potential, but it required a more negative potential. In fact, the rate of the reduction at a potential more positive than −1.0 volt on platinum cathode was very slow, and it seemed that cytochrome c might be reduced by the action of hydrogen which was produced by the hydrolysis of water.

The absorption spectrum of the electrolytically reduced cytochrome c was the same with that obtained by the action of dithionite, but it was not fully active in respect to the action of cytochrome a_3. The inactivation might be caused by the denaturation of the protein moiety of cytochrome c, because it was known that the S−S linkage was reduced electrolytically.

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A Diphosphopyridine Nucleotide Oxidase in Mung Bean Seedlings*

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Although the electrolytically obtained DPNH was not completely oxidized by usual dehydrogenases or diaphorases, one of the authors noticed that its absorption band at 340 mμ disappeared completely when it was incubated with the extract of mung been seedlings. The reaction was found to be stimulated by the addition of methylene blue, and the product was identified as DPN. Thus, the reaction resembled that of diaphorase, although it was less specific to the configuration of DPNH. But unlike usual diaphorase, it required a cofactor, which was neither flavins nor metallic ion, but an unidentified acidic substance. General properties of the enzyme and the cofactor are reported in this article.

I. INTRODUCTION

It was reported in previous papers[1,2] that electrolytically reduced diphosphopyridine nucleotide (DPNH) was only partially oxidized by alcohol-, malic acid-, or lactic acid dehydrogenase system. It was also only partially oxidized by diaphorases from yeast or pig heart, but was completely oxidized by the water extract of etiolated mung bean seedlings.

Thus, the extract of mung bean seedlings is supposed to contain a diaphorase which is less specific to the configuration of DPNH, because the electrolytically obtained dihydrodiphosphopyridine nucleotide (DPNH) is a mixture of the following two isomers shown in Fig. 1, in which I is active enzymatically, while II and III are inactive.

Actually, the rate of the oxidation of DPNH by the extract of the seedlings were stimulated by the addition of methylene blue similar to the reaction of other diaphorases. But unlike the other diaphorases, this enzyme required a cofactor which was neither of cation, nor flavin, nor ascorbic acid.

The cofactor is an acid still unidentified which is relatively stable in acid or alkali even at 100°C and resistant to ultraviolet-irradiation. It is soluble into phenol, but does not transdissolve either into ether or chloroform even from acidic water solution.

The protein moiety of the enzyme was rather unstable, especially in alkaline solution. It was purified to some extent by differential precipitation with ammonium sulfate and

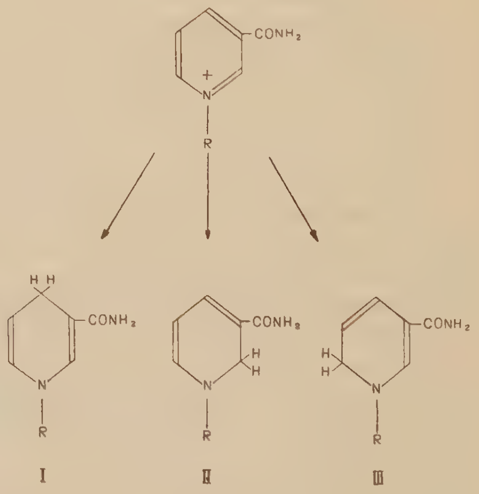

FIG. 1.

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* Electrolytic Reduction of Physiologically Important Substances at Controlled Potentials. Part III.

1) T. Kôno, This Bulletin, **21**, 115-120 (1957).

2) T. Kôno and S. Nakamura, *ibid*., **22**, 399~403 (1958).

electrophoresis.

General properties of the enzyme and its cofactor are presented.

II. MATERIALS AND REAGENTS

1) Mung bean seedlings: Etiolated mung bean seedlings from 3 to 5 days old were used.

2) Crude enzyme extract: Mung bean seedlings were homogenized below 5° with the addition of one half of its weight of 0.1 M Na_2HPO_4 solution. The homogenate was squeezed out through a sheet of cloth and the solution was centrifuged at 12,000 × g for 15 min. The supernatant solution was used as a crude enzyme extract. As the enzyme was rather unstable in solution, the solution was saturated with ammonium sulfate and kept in an ice box. The precipitated enzyme could be kept at −5° for a week without much loss of activity.

3) DPN: Diphosphopyridine nucleotide was purchased from Nutritional Biochemicals Co., U.S.A.

4) DPNH: DPNH was prepared by reducing DPN with either sodium dithionite or by electrolytic reduction at a controlled potential. The apparatus and the conditions for the reduction were the same with those reported in the previous paper[1], except that 0.1 M tris(hydroxymethyl) aminomethane (tris) buffer, pH 7.4, was used in this experiment. The concentration of DPNH was controlled so that the optical density of the solution at 340 mμ was about 0.300 at the beginning of each experiment.

III. GENERAL METHODS

The activity of the enzyme was determined by measuring the decrease of optical density at 340 mμ. A Hitachi spectrophotometer was used. The composition of the reaction mixture was as follows:

DPNH	0.15 μ mole
methylene blue	1 μ mole
enzyme solution	adequate amount.
0.1 M tris buffer, pH 7.4	3 ml

The solutions were pipetted in the cell and the decrease of optical density at 340 mμ was measured every 15 seconds beginning at 1 minute after the mingling. The decrease of optical density in the course of 2.5 mins. was taken as the activity of the enzyme. The concentration of the enzyme was controlled so that the decrease of the optical density in 2.5 mins. was less than 0.050 because the rate of decrease in optical density was linear in such a condition.

The amount of protein in the solution was measured by Folin's method modified by Lowry et al.[3], and expressed as the amount of egg albumin.

IV. EXPERIMENTS AND RESULTS

A. General properties of the enzyme.

1. Optimum pH of the reaction. The optimum pH of the reaction was 7.4, as shown in Fig. 2.

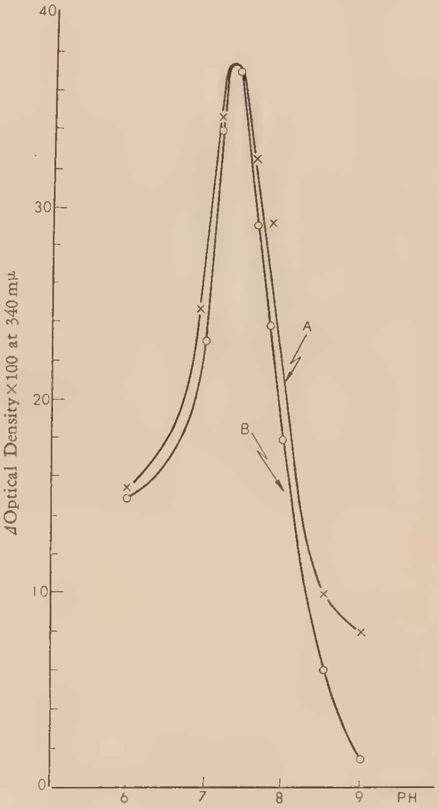

FIG. 2. Activities of the Enzyme at Different pH Values.

A: DPNH prepared with dithionite was used as the substrate, B: DPNH prepared electrolytically was used.

2. Effects of activators and inhibitors. Although the crude enzyme extract itself could oxidize DPNH, the addition of methylene blue stimulated the reaction (Table I). But the addition of cytochrome c was ineffective. The reaction was not appreciably inhibited by 10^{-4} M of *p*-chloromercuribenzoate, KCN, 2,4-dinitrophenol, or NaN_3.

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[3] O. H. Lowry, N. J. Rosenbrough, A. L. Forr and R. T. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

TABLE I. EFFECTS OF ACTIVATORS AND INHIBITORS

	Materials	concn.(M)	activity
1.	crude enzyme extract*		18
2.	boiled enzyme extract		0
3.	1+cytochrome c	10^{-5}	15
4.	1+methylene blue	10^{-5}	50
5.	2+methylene blue	10^{-5}	13
6.	4+*p*-mecurichlorobenzoate	10^{-3}	47
7.	4+KCN	10^{-3}	49
8.	4+2, 4-dinitrophenol	10^{-4}	45
9.	4+NaN_3	10^{-4}	50
		10^{-3}	45

* 0.2 ml of five times diluted crude enzyme extract was used in each experiment.

3. The product of the reaction. The product of this enzymatic reaction was intact DPN, because the absorption band around 340 $m\mu$, which is specific to DPNH, was fully visible on reducing the product by the action of alcohol dehydrogenase system, Fig. 3.

The experiments were performed in the following way: Electrolytically obtained DPNH in tris-buffer, pH 7.4, with an optical density of 0.680 at 340 $m\mu$, was incubated with 0.5 ml of the crude enzyme extract for 30 min. without the addition of methylene blue. The solution was adjusted to pH 9.0 by adding 1 N NaOH solution, and treated with alcohol and alcohol dehydrogenase as usual, using semicarbazide to trap acetaldehyde[4]. The original DPNH solution, oxidized solution, and enzymatically reduced solution were filled up to the same volume with 0.1 M tris-buffer pH 7.4, and their absorption spectra were determined.

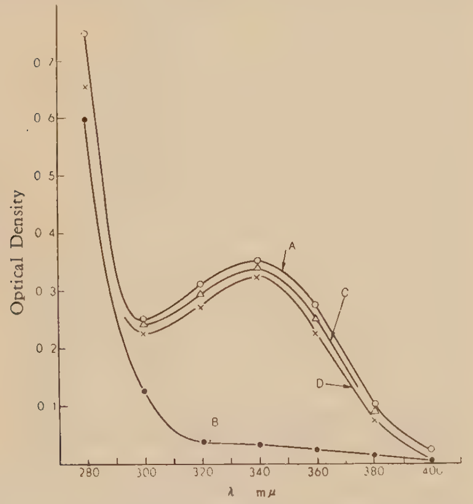

FIG. 3. The Oxidation of Electrolytic EPNH with the Extract of Mung Bean Seedlings Followed by the Reduction with Alcohol Dehydrogenase System.

A: ○——○ Electrolytic DPNH, B: ●——● A. was oxidized with the extract of mung bean seedlings, C: ×——× B. was reduced by alcohol dehydrogenase system, D: △——△ B. was reduced with sodium dithionite.

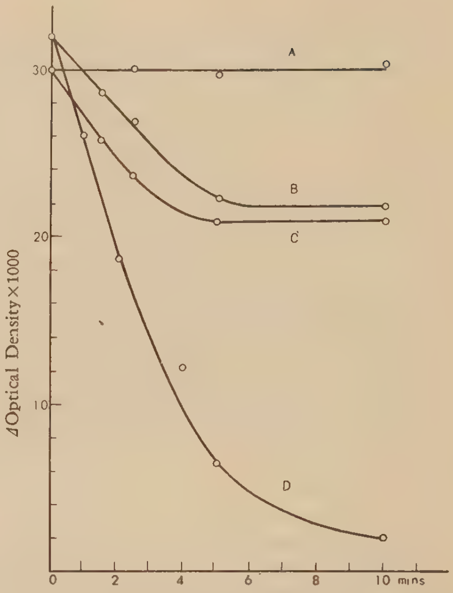

FIG. 4. Oxidation of Electrolytic DPNH with, A: boiled extract of mung bean seedlings, B: yeast diaphorase, C: pig heart diaphorase, D: crude extract of mung bean seedlings.

4. Specificity of the enzyme. As it was indicated in the previous report, the crude enzyme extract of mung bean seedlings could oxidize electrolytically obtained DPNH completely, as well as DPNH reduced by dithionite. The rates of the oxidation were the same, and the optimum pH was also identical, Fig. 2. The action of the enzyme was the same with that of diaphorase obtained from other origins. But, as it is shown in Fig. 4, diaphorases from yeast or pig heart could not oxidise the electrolytically obtained DPNH completely.

Therefore, it may be concluded, that the enzyme in mung bean seedlings have a diaphorase activity, but it is not specific to the configuration of pyridine moiety of DPNH, assuming that the difference of the two kinds of DPNH was due to the different configurations

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4) P. K. Bonnichsen: *Acta. Chim. Scand.*, **4**, 714 (1950).

in the pyridine ring.

The enzyme in solution was rather labile at room temperature, especially in alkaline medium. It was destroyed almost instantaneously above pH 9.0, at room temperature. The heat stabilities of the enzyme are illustrated in Table II. The data again indicate that there exists one enzyme which is not specific to two kinds of DPNH, because heat stabilities of the activities to the two kinks of DPNH are almost identical.

TABLE II. HEAT STABILITY OF THE ENZYME

Temperature °C	Time	Activity remained%	
		DPNH-elect.	DPNH-chem.
0	0 mins.	100	100
50	5 〃	70	70
60	5 〃	60	63
70	5 〃	52	48
80	5 〃	0	0
0	24 hrs.	73	—
20	24 〃	63	—

B. Purification of the protein moiety of the enzyme which requires a cofactor

1. Requirement for a cofactor. It was observed that the protein fraction of the crude enzyme extract, which was precipitated from 50% alcohol solution or from a saturated ammonium sulfate solution, showed only from one-tenth to one-fifth of the original activity when it was filled up to the original volume. But activity was fully recovered when the boiled crude extract was added to the reaction mixture. These relationships are illustrated in Table III.

TABLE III. REQUIREMENT FOR A HEAT-STABLE COFACTOR

	Materials	Activity
1.	crude enzyme extract*	50
2.	ppt. from a saturated $(NH_4)_2SO_4$ soln.**	5
3.	2+boiled enzyme extract***	55
4.	ppt. from a 50% alcohol soln.**	10
5.	4+boiled enzyme extract	55

* Ten ml of the original solution was filled up to 50 ml, and 0.2 ml of the solution was used.

** Precipitates from 10 ml of the original solution was filled up to 50 ml, and 0.2 ml of the solution was used.

*** Ten ml of the original solution was boiled for 10 mins. and centrifuged. The supernatant was filled up to 50 ml and 0.2 ml of the solution was used.

The rate of the oxidation of DPNH obtained by the reduction with dithionite was also depressed by the absence of the cofactor. The rates of the oxidation of both DPNH, obtained electrolytically and chemically, by a protein-fraction precipitated from a saturated ammonium sulfate solution of crude enzyme extract are illustrated in Table IV.

TABLE IV. RATES OF OXIDATION OF TWO DPNH BY THE PROTEIN FRACTION*

	DPNH-elect.	DPNH-chem.
1	30	31
2	32	33
3	35	34
4	31	32
mean	32.0	32.5

* The concentration of the protein fraction is about 5 times as much as in the case of Table II.

2. Fractionation of enzyme protein. Because the protein moiety of the enzyme was found to require a cofactor, or several cofactors, which was present in the boiled extract of mung bean seedlings, all determinations in this section were performed with the addition of the boiled extract in the reaction mixture.

Most of the total activity was precipitated by saturating the crude enzyme extract with ammonium sulfate (See Table V in the next section). The specific activity was doubled by the above treatment, but no critical concentration of ammonium sulfate to precipitate the activity was observed.

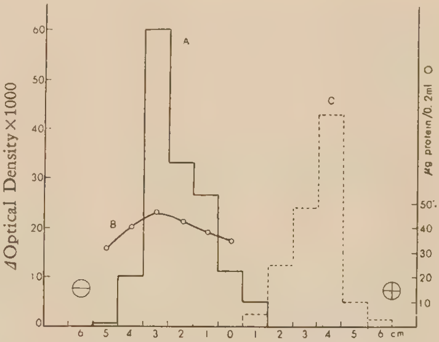


FIG. 5. Electrophoresis of the Protein and the Cofactor of the Enzyme.

A & B: Electrophoresis of the protein, which was precipitated with ammonium sulfate. A. indicates the distribution of the activity and B. indicates the distribution of the protein. C: Electrophoresis of the cofactor, which was obtained by phenol extraction. A& B and C are different experiments, althouth they are shown in a figure.

Addition of an equal volume of alcohol or acetone to the crude enzyme solution at −2°, followed by dialysis below 5°, easily insolubilized the enzyme. The insolubilized enzyme, however, showed the activity to be almost comparable to the soluble one.

3. Electrophoresis of the enzyme protein. An enzyme solution which had a specific activity about ten times as much as the original solution, was obtained by the electrophoresis of the ammonium sulfate precipitate of the enzyme, Fig. 5−A and −B.

The experimental conditions were as follows. Crude enzyme extract was obtained from 100 g of mung bean seedlings. The extract was saturated with ammonium sulfate, and the precipitate was dissolved into 20 ml of 0.1 M tris-buffer, pH 7.4, and 2 ml of the solution was used in the electrophoretic separation. The electrophoretic column was a horizontal starch column of 30 cm in length, 4 cm in width, and 1 cm in thickness. The electrophoretic solution was 0.1 M tris-buffer, pH 7.4, the electric current was about 20 mA, and the voltage difference was about 7 volts per cm. The electrophoresis was continued for 20 hr. at 0–4°. Then, the starch column was cut at every 1 cm, and the pieces were eluted with 10 ml of tris buffer solutions.

A typical example of the purification is shown in Table V.

TABLE V. PURIFICATION OF THE ENZYME PROTEIN

	Total activity %	Sp. activity*	Sp. act. %
Crude enzyme extract	100	0.030	100
ppt. from satd. $(NH_4)_2SO_4$	79	0.064	213
Electrophoretic fraction No. 3	?	0.344	1143

* Specific activity was expressed as the decrease of the optical density at 340 mμ/min./mg. protein.

C. The nature of the cofactor.

1. Several characteristics of the cofactor. Several characteristics of the cofactor are summarized in Table VI.

These data indicate that the cofactor is neither flavins, nor metallic ions, but is a compound rather stable to heat both in alkaline and acidic medium. It is also resistant to ultraviolet-light irradiation. The crude cofactor solution absorbed ultraviolet-light intensively, but no absorption peak was observed in either ultraviolet or visible region of the spectrum.

2. Separation of the cofactor by solvents and ion exchange resins. Mung bean seedlings were homogenated in alcohol, and the extract was fractionated as follows. Table VII.

The data in Table VII indicate that the cofactor is an acid which is not soluble into chloroform even from acidic aqueous solution. The cofactor is soluble in phenol saturated with water.

TABLE VI. NATURE OF THE COFACTOR

			Activity
1.	crude enzyme extract		50
2.	protein fraction		7, 5
3.	cofactor solution		0
4.	2+3		53, 55
5.	2+U. V. irradiated 3		45, 53
6.	2+3, treated in alkaline soln.		53, 54
7.	2+3, treated in acidic soln.		50, —
8.	2+riboflavin	10^{-3} M	8, 5
9.	2+flavin mononucleotide	10^{-3}	6, 8
10.	2+flavin adenine dinucleotide	10^{-3}	10, 7
11.	2+ash of mung bean as sulfate		18, 12
12.	2+ash+FAD		16, 10
13.	2+ascorbic acid	10^{-2}	9, 7
14.	2+cytochrome c	10^{-5}	6, 8

Remarks:
1. Crude enzyme extract from 10 g of mung bean seedlings was filled up to 50 ml.
2. Protein fraction, from 10 g of seedlings, pptd. by $(NH_4)_2SO_4$, was filled up to 50 ml.
3. Alcohol extract of 10 g of the seedlings was dried up in vacuo, and filled up to 50 ml.
4. Equal volumes of (2) and (3) were added.
5. Dried up cofactor, 3, was filled up to 10 ml, 10 ml of 1-N NaOH was added, irradiated with U.V. light for 1 hr, neutralyzed with 1-N HCl, and filled up to 50 ml.
6. Instead of the irradiation in 5, the alkaline soln. was dipped into a boiling water bath for 1 hr.
7. The same with 6, using HCl instead of NaOH, and vice versa.

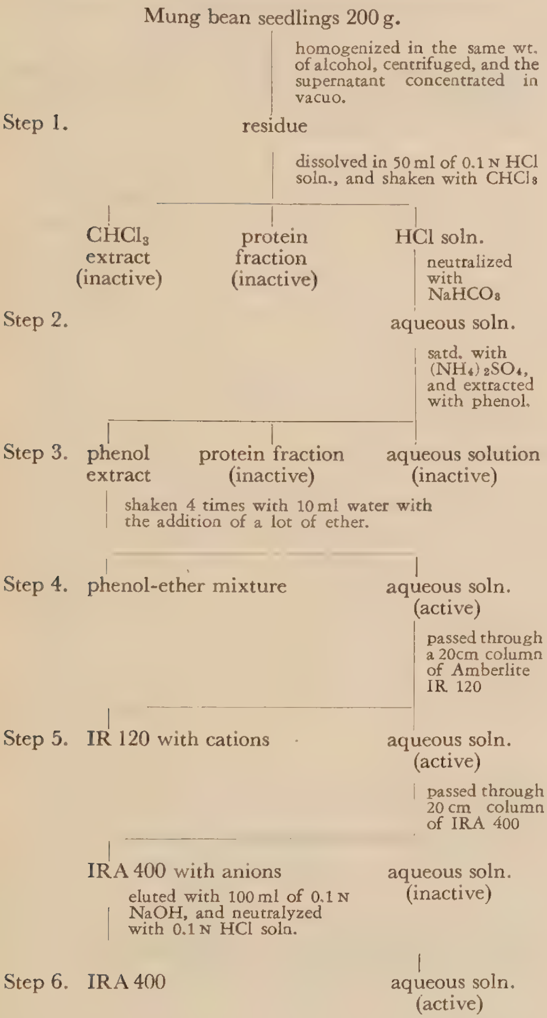
The elution of the cofactor from Amberlite IRA 400 resin with different concentrations of HCl and NaCl solution was not successful.

3. Electorophoretic separation of the cofactor. The cofactor transdissolved from phenol to water (Step 4 in Table VII) was further purified by an electrophoresis using the horizontal starch column used for the purification of protein moiety (Section B). The electrophoretic conditions were the same as those used for the separation of protein, except that the time was 3 hr. The activities of the fractions were measured using the protein fraction of No. 3 in Fig. 5–A. The distribution of activity on the column is illustrated in Fig. 5–C.

V. DISTRIBUTION OF THE ENZYME AND THE COFACTOR

The distribution of the enzyme in different parts of mung bean seedlings was determined as shown in Table VIII. The data indicate that the enzyme is distributed

TABLE VII. SEPARATION OF THE COFACTOR



Mung bean seedlings 200 g.

homogenized in the same wt. of alcohol, centrifuged, and the supernatant concentrated in vacuo.

Step 1. residue

dissolved in 50 ml of 0.1 N HCl soln., and shaken with $CHCl_3$

$CHCl_3$ extract (inactive) | protein fraction (inactive) | HCl soln.

neutralized with $NaHCO_3$

Step 2. aqueous soln.

satd. with $(NH_4)_2SO_4$, and extracted with phenol.

Step 3. phenol extract | protein fraction (inactive) | aqueous solution (inactive)

shaken 4 times with 10 ml water with the addition of a lot of ether.

Step 4. phenol-ether mixture | aqueous soln. (active)

passed through a 20cm column of Amberlite IR 120

Step 5. IR 120 with cations | aqueous soln. (active)

passed through 20 cm column of IRA 400

IRA 400 with anions | aqueous soln. (inactive)

eluted with 100 ml of 0.1 N NaOH, and neutralyzed with 0.1 N HCl soln.

Step 6. IRA 400 | aqueous soln. (active)

TABLE VIII. DISTRIBUTION OF THE ENZYME IN MUNG BEAN SEEDLINGS*

	Etiolated for 7 days			Etiolated green	
	epicotyl	hypocotyl	root	2 days	7 days
Fresh weight (g)	16	56	15	—	—
Activity**	125	30	100	32	22
Protein***	1700	305	350	400	390
Sp. activity****	37	49	143	40	28

* Mung bean seedlings were extracted with five times as much buffer solution as its fresh weight. The activity and the amount of protein in 0.1 ml of the extract were illustrated. More diluted solutions were used and the data were calculated in the first and the third columns.

** Difference of the optical densities ×1000/2 mins./0.1 ml.

*** μg protein/0.1 ml.

**** Difference of the optical densities ×1000/min./mg protein.

in every part of the plant body of etiolated seedlings. The enzyme is also present in green seedlings grown in the light, but the amount is less than that in the etiolated ones.

The distribution of the enzyme in the cells of mung bean seedlings was determined by homogenizing the seedlings in a solution containing 0.05 M tris buffer and 0.5 M sucrose, followed by differential centrifugation at low temperature⁵⁾. The fractions, a) which precipitated at 4000 × g for 5 min, b) which precipitated between 4000 – 12,000 × g, and c) the supernatant were obtained. The fraction a) was inactive, whereas fractions b) and c) showed almost the same specific activities, indicating that the enzyme was not exclusively present in mitochondria, but rather distributed in cytoplasm.

Diaphorase which could oxidize electrolytically obtained DPNH completely was not found in spinach, turnip, yeast, and heart muscle.

The distribution of the cofactor was also studied using the electrophoretically purified protein moiety of the enzyme, but again, no activity could be detected in the extracts of spinach, turnip, yeast, liver and heart muscle of a pig.

VI. DISCUSSION AND CONCLUSION

As it had been observed by Lockhart⁶⁾, a diaphorase activity was found in mung bean seedlings.

The enzyme, however, differs from the other enzyme in two characters. The one is that the enzyme can completely oxidize both kinds of DPNH obtained by dithionite and electrolytic reduction, while the other diaphorases or dehydrogenases are not able to oxidize the latter completely. The oxidation product of the enzyme is DPN, because it can be re-reduced by alcohol dehydrogenase-system.

The second characteristic of the enzyme is that it requires a cofactor, or cofactors, in the reaction. The cofactor is an acid which is heat stable, resistant to 0.1 N NaOH and 0.1 N HCl at 100° for 1 hr., also resistant to ultraviolet-

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5) T. E. Humphreys and Eric E. Conn, *Arch. Biochem. Biophys.*, **60**, 226-243 (1950).

6) E. E. Lockhart, *Biochem. J.*, **33**, 613 (1939).

irradiation in alkaline medium, soluble to phenol, but not to ether or chloroform from aqueous solution even at pH 1. It is neither riboflavin, nor flavin mononucleotide, nor flavin dinucleotide, nor ascorbic acid, nor metallic ion in the seedlings.

The identification of the cofactor is a question open for further investigation.

The author is thankful to Prof. S. Funahashi and Assoc. Prof. M. Nakamura for their interest in this research.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 6, p. 410~419, 1958]

Fat Synthesis in Unicellular Algae

Part III. Absorption of Nitrogen by Nitrogen-deficient Chlorella Cells and its Effects on the Continuous Cultivation of Fatty Cells

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Received June 16, 1958

The effects of supplying a small amount of nitrogen to nitrogen-deficient Chlorella cells were briefly investigated. Stimulation of both respiration and photosynthesis were observed with various inorganic nitrogen compounds and urea. The stimulation of respiration disappeared, when all of the nitrogen supplied had been absorbed, but that of photosynthesis lasted, after all of the nitrogen had been absorbed.

The effect of this kind of stimulation of photosynthesis on the growth of nitrogen-deficient cells was investigated by semi-continuous and continuous cultivations. The growth was stimulated by nitrogen absorption, and it was well established that the continuous cultivation can be carried out for a certain long period with a much better yield than was expected, owing to this stimulatory effect on growth, in spite of the progressing chlorosis in stronger light intensity. The crops changed their fat content slightly in the course of cultivation.

It was reported previously[1] that Chlorella grown in a complete medium can grow farther when transferred into a nitrogen-free medium, accumulating lipid in the cells. Nitrogen-deficient cells thus obtained assimilate nitrogen freshly supplied to the medium at a very high rate, and in this occasion interesting phenomena i.e., the stimulations of respiration and photosynthesis occur.

As for the stimulatory effect of nitrogen assimilation on respiration of nitrogen-deficient cells, we have Pirson's classical work[2]. He observed the stimulation of respiration with both potassium nitrate and ammonium sulphate, but that of photosynthesis only with the former. He attributed the stimulation of photosynthesis by the former to the effect of potassium ions. Syrett[3] studied later on the stimulation of respiration extensively but not that of photosynthesis.

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[1] H. Iwamoto, G. Yonekawa and T. Asai, This Bulletin, **19**, 240 (1955).

[2] A. Pirson, *Z. Bot.*, **31**, 193 (1937).

The authors observed the stimulatory effect on photosynthesis with both compounds, and concluded that the effect results from nitrogen assimilation. This kind of investigation is interesting in connection with the mechanism through which nitrogen-deficiency affects photosynthesis and also to the practical problem of growing nitrogen-deficient Chlorella cells by continuous cultivation for the purpose of producing fat.

Many investigations have been reported on the continuous cultivation of yeast, and some on that of Chlorella and bacteria, from the point of view of its academic or industrial interest. As for Chlorella, Cook[4], Myers[5], and Fujimoto et al[6] reported successful operations of the continuous cultivation of normal cells. And the semi-continuous harvest was considered to be most rational in the practical cultivation of normal cells[6].

On the other hand, the continuous cultivation of nitrogen-deficient fatty cells is quite a different problem, because in nitrogen-dificiency yeast and Chlorella show entirely different growth curve from that in the normal condition. Since the higher the degree of nitrogen deficiency of Chlorella cells, the smaller the photosynthetic activity and consequently the growth rate of the cells, it is quite natural to conceive that the yield of highly nitrogen-deficient fatty cells may be very small in a continuous cultivation, unless a special kind of stimulation of growth is induced by nitrogen assimilation in nitrogen-deficient cells.

In this report, these various problems are investigated and the possibility of producing fat by the continuous cultivation of Chlorella is discussed, beginning with a brief study on the patterns of nitrogen assimilation by nitrogen-deficient cells.

MATERIAL AND METHODS

The organism used was Chlorella ellipsoidea. The composition of the medium was the same as previously described[1]. The culture apparatus used for the seed culture and semi-continuous culture was the same as illustrated in the same paper, and that for the continuous culture was also the same as illustrated in another paper[6].

Normal cells grown in the complete medium were collected, washed with distilled water, and resuspended in the nitrogen-free medium at the algal concentration of about one gram per litre, and illumination with a 300 W spot light and aeration with CO_2 enriched air were started again. After several days the cells were collected and used as nitrogen-deficient cells.

The rate of respiration and the respiratory quotient were determined by Warburg's direct method, and the rate of photosynthesis and the photosynthetic quotient were by Warburg's indirect method. The rate of respiration in the light was assumed to be the same as that in the dark according to Brown[7].

Operations of semi-continuous (intermittent) or continuous feeding and harvest were started when a certain degree of nitrogen-deficiency of the cells was attained. In the semi-continuous culture, one-fifth of the whole culture was removed and the fresh medium of the same volume was added once a day at a fixed time. The fresh medium added was made up so as to contain just the same amount of nitrogen in the form of potassium nitrate as that removed in the form of cell-nitrogen by harvest. In the continuous culture, the fresh medium which had the same concentration of nitrogen as the whole algal suspension was added to the cultnre drop by drop, and the suspension was removed by overflow.

Methods of determination of algal conentration, total nitrogen and lipid contents of cells were the same as previously described[1]. Soluble organic nitrogen compounds in the cells were extracted with 0.1% trichloracetic acid solution four times at 80°C for 15 minutes, after being extracted with the same solution at 100°C for 1.5 minutes. All the extracts were combined, and the nitrogen was determined by the semi-micro Kieldahl method. Extraction and determination of carbohydrate fractions were pursued after the description in the previous paper[8].

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[3] P. J. Syrett, *Ann. Bot.*, **17**, 1 (1953).

[4] R. M. Cook, *Ind. Eng. Chem.*, **43**, 2385 (1951).

[5] R. L. Myers and L. B. Clark, *J. of Gen. Physiol.*, **28**, 193 (1944).

[6] Y. Fujimoto, H. Iwamoto, A. Kato and K. Yamada, This Bulletin, **20**, 13 (1956).

[7] A. H. Brown, *Amer. J. Bot.*, **40**, 719 (1953)

Extraction of chlorophyll was pursued as follows; ten ml of algal suspension containing 30–60 mg of cells in dry weight was centrifuged, and the cells were extracted with five ml of methanol several times and finally with five ml of ethyl ether. All the extracts were combined and the methanol was washed down in a separation funnel with water. The ether was dehydrated with Glauber's salt and was made up to 25 ml. This chlorophyll solution was subjected to the determination of chlorophyll with a spectrophotometer. The total chlorophyll was calculated by the following equation[9]. Total chlorophll (mg/l)$=7.12\log I_0/I$ (at 6600Å)$+16.8\log I_0/I$ (at 6425 Å).

RESULTS AND DISCUSSIONS

Assimilation of nitrogen by nitrogen-deficient cells.

Active assimilation of nitrogen occurs both in the dark and in the light, when nitrogen-deficient cells are supplied with a assimilable nitrogen compound. Fig. 1 shows the absorption curves when plenty of ammonium sulphate was supplied to the nitrogen-deficient cells of about 3% nitrogen content. In the light, the cells recovered from their nitrogen-deficiency, when CO_2 was supplied, but in the dark, the nitrogen-deficiency was not completely recovered. Fig. 1 and Table I show that the nitrogen content increased from about 3% to 4% and from 3.62% to 5.93% respectively in the dark.

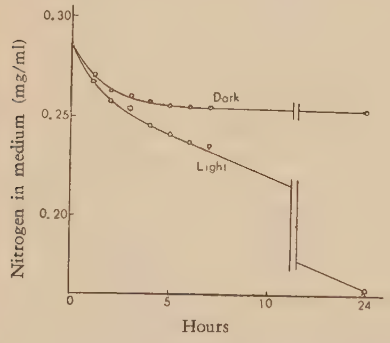

FIG. 1. Nitrogen Absorption by Nitrogen-deficient Cells.

The initial and final nitrogen content of the cells were about 3 and 4% respectively in the dark.

The velocity of absorption was very rapid in the very early phase of absorption both in the light and in the dark, and difference between them was scarcely observed. Fig. 1 shows that in both cases the cells absorbed nitrogen equivalent to about 0.3% of their dry weight in half an hour.

The nitrogen absorbed in the dark was mainly accumulated as soluble nitrogen compounds as shown in Table I. Almost all of the nitrogen absorbed appeared once as soluble nitrogen, and were then gradually converted into insoluble nitrogen. Consequently, the percentage of soluble nitrogen in total nitrogen increased very rapidly at first, and reached about 40%, and then decreased very slowly. This large percentage decreased rapidly when glucose was supplied to the cells. This fact, together with the fact that vigorous nitrogen absorption occurs again when the cells which have stopped absorbing nitrogen any more are supplied with glucose, suggests that nitrogen assimilation and conversion of soluble nitrogen into insoluble one require glucose or similar metabolical carbon reserves. It is natural to consider that the cessation of nitrogen assimilation and conversion into insoluble fraction is due to the exhaustion of metabolical carbohydrates in the cells accompanied with

TABLE I. CHANGES OF SOLUBLE N OF N-DEFICIENT CELLS IN N-ASSIMILATION IN THE DARK.

Time hr.	N absorbed	Soluble N	Increase in sol. N (% in N absorbed)
0	0	17.7	0 (0)
0.5	8.8		
1	11.1		
2	18.8	35.6	17.9 (95.2)
3	30.5	47.3	29.6 (97.0)
8	32.7	42.1	24.4 (74.6)
11	34.5	42.1	24.4 (70.7)
15.5	37.2		
21	41.4		

The initial and final algal density and N-content of the cells are 2.37 g/l and 2.07 g/l, 3.62% and 5.93%, respectively.

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8) H. Iwamoto and N. Nagahashi, This Bulletin, **19**, 247 (1955).

9) Official Method of Analysis of the A.O.A.C., p. 113 (1950).

the nitrogen assimilation[10].

As shown in Table 2, remarkable decrease was observed in the contents of free reducing sugar, sucrose and water soluble polysaccharide, after nitrogen assimilation had almost stopped.

TABLE II. CHANGES OF CARBOHYDRATE FRACTIONS OF N-DEFICIENT CELLS BEFORE AND AFTER N-ASSIMILATION IN THE DARK
(percentage in the dry weight)

	Before	After
Free reducing sugar (as glucose)	2.82	1.54
Sucrose (as sucrose)	3.72	1.15
Oligosaccharide* (as glucose)	1.50	1.72
Water-soluble polysaccharide (as glucose)	2.30	1.07

* The net amount of this fraction remained unchanged.

But complete exhaustion of any of these fractions was not seen. It is clear that rapid assimilation does not always continue until the carbon reserves of the cells are completely exhausted. And also, it seems that fat is not metabolized readily, because the nitrogen-deficient cells did not show a respiratory quotient lower than unity even after sixteen days of the starvation period in the dark.

Stimulation of respiration in nitrogen-deficient cells by nitrogen assimilation.

It was reported by several investigators that the stimulation of respiration occurs when nitrogen-deficient cells of Chlorella[2,3] and Serratia marcescens[11,12] and excised barly root[13] are supplied with ammonium sulphate or some other nitrogen compounds. The authors also observed the same kind of stimulation with potassium nitrate, hydroxylamine and urea as well as ammonium sulphate as shown in Figs. 2, 3 and 4. In the case of ammonium sulphate, the maximum rate of oxygen absorption was observed instantly after it was supplied, while in the cases of the other three, a lag was observed before the

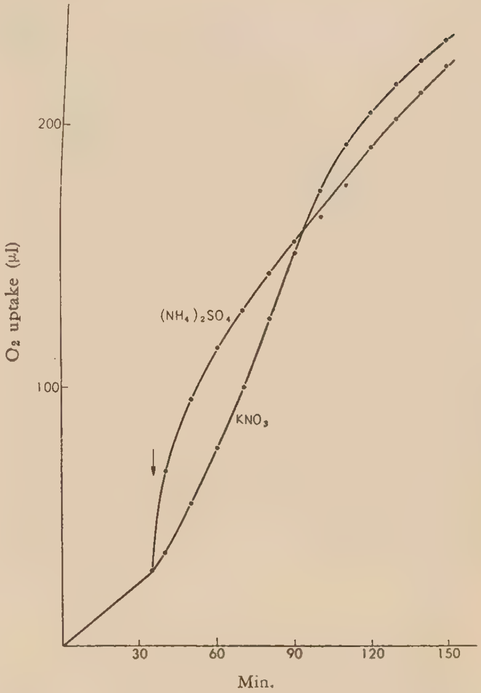

FIG. 2. Stimulation of Respiration by $(NH_4)_2SO_4$ and KNO_3 in Nitrogen-deficient Cells.

Cells used: ca. 30 mg, N-content of the cell: 2.9%, Nitrogen added: 26.5 γ.

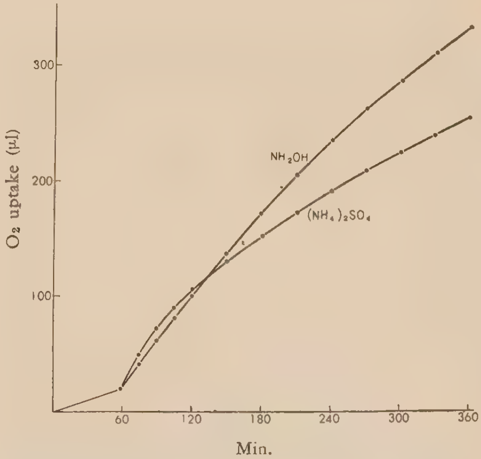

FIG. 3. Stimulation of Respiration by $(NH_4)_2SO_4$ and NH_2OH in Nitrgen-deficient Cells.

Cells used: ca. 30 mg, N-content of the cells: 2.4%, Nitrogen added: 28 γ.

10) P. J. Syrett, *Ann. Bot.*, **17**, 21 (1953).
11) D. T. McLean and K. C. Fisher, *J. Bact.*, **54**, 599 (1947).
12) D. T. McLean and K. C. Fisher, *J. Bact.*, **58**, 417 (1949).
13) A. J. Willis, *Biochem. J.*, **49**, 27 (1951).

maximum rate of oxygen absorption was reached. The lag in the case of nitrate might be due to the fact that it must be reduced to ammonium ions before it is assimilated. The lag in the case of urea must be further investigated along with the mechanism of the assimilation of urea, if it is true that urease does not exist in Chlorella cells[14].

The stimulation disappeared when all of the nitrogen supplied had been absorbed, and it is interesting to find that the amount of oxygen absorbed per μmol of the nitrogen assimilated was almost the same in each compound except hydroxylamine. In the case of hydroxylamine, the amount of oxygen was very large compared with the cases of the other compounds, which suggests that further investigation concerning the cause of the stimulation by hydroxylamine is necessary.

The respiratory quotient dropped temporarily and then recovered in both cases of ammonium sulphate and urea. The dropping degree of R.Q. was larger in the former case than in the latter case. This might be due to the release of carbon dioxide in the course of urea assimilation, even if urea is not assimilated through urease. In the case of nitrate, R.Q. became higher at first and then dropped lower than the initial. This might be due to the release of "extra carbon dioxide" in the earlier phase, owing to the oxidation of extra carbohydrate for the reduction of nitrate[15], and to the successive assimilation of ammonium ions in the later phase.

Stimulation of photosynthesis in nitrogen-deficient cells by nitrogen assimilation.

Stimulation of photosynthesis also occurred along with that of respiration. Fig. 5 shows the stimulation caused by the supply of ammonium sulphate. The rate of carbon dioxide assimilation was doubled after the stimulation

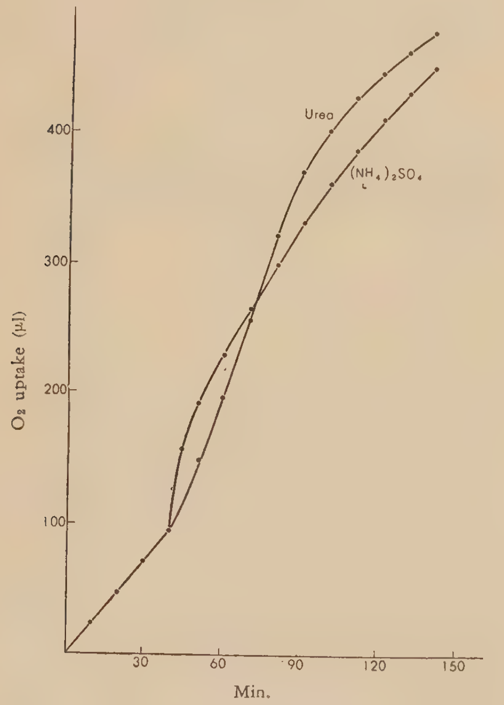

FIG. 4. Stimulation of Respiration by $(NH_4)_2SO_4$ and Urea in Nitrogen-deficient Cells.

Cells used: ca. 50 mg, N-content of the cells: 3.0%, Nitrogen added: 55 γ.

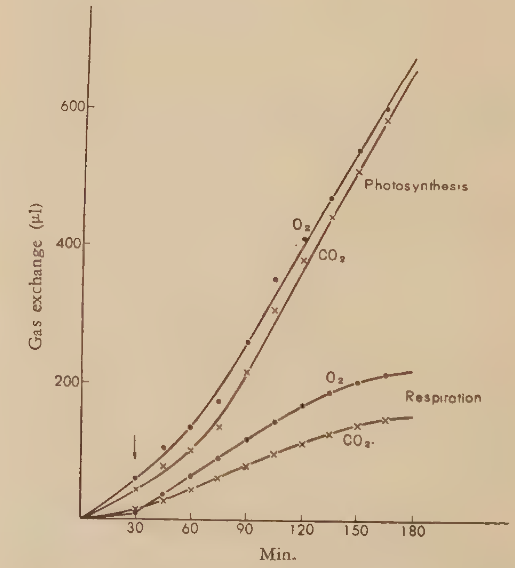

FIG. 5. Stimulation of Photosynthesis by $(NH_4)_2SO_4$ in Nitrogen-deficient Cells.

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[14] J. B. Walker, *Proc. Natl. Acad. Sci. U.S.*, **38**, 561 (1952).

[15] O. Warburg and E. Negelein, *Biochem. Z.*, **110**, 66 (1920).

occurred. The stimulation continued even after all of the nitrogen was absorbed, while the stimulation of respiration disappeared. A lag phase of about 30–45 minutes was observed before the stimulation occurred. It is not clear what kind of factor is the cause of the stimulation, but the factor is distinctly made by the assimilation of a very small amount of nitrogen and remains after the assimilation ceases. According to Pirson[2], the stimulation did not occur in the case of ammonium sulphate, occurring only in the case of potassium nitrate. Hence Piron attributed the phenomenon to the stimulatory effect of potassium ions. But this does not seem to be so. Although it is clear to the authors that the stimulation is caused by nitrogen assimilation, it is also clear that it is not caused by the increase in chlorophyll content of the cells, because the chlorophyll content did not increase even after two hours of nitrogen assimilation as shown in Table III. Fleischer[16] described that the capacity for photosynthesis of cells of Chlorella which were made chlorotic by nitrate deficiency was proportional to their content of chlorophyll. However, it can be concluded from the above results that there is some factor other than chlorophyll which controls photosynthesis primarily in the case of nitrogen-deficiency and is highly activated by slight nitrogen assimilation. This will be discussed again later on.

TABLE III. CHANGES OF CHLOROPHYLL CONTENT AFTER N-ASSIMILATION IN N-DEFICIENT CELLS
(mg/cells in 1 litre of suspension)

Initial	After 2 hours	
	Light	Dark
7.53	6.48	6.57

Stimulation of growth of nitrogen-deficient cells by nitrogen assimilation.

It is quite reasonable to presume that the stimulation of photosynthesis will affect the growth rate and give a good effect on the continuous cultivation of nitrogen-deficient cells, because in that cultivation nitrogen-deficient cells are kept in such a state as assimilating a very small amount of nitrogen constantly.

A typical nitrogen-free growth of Chlorella is shown by the curve AB in Fig. 6. If a portion of the culture is removed and the same volume of fresh medium is added at C, algal density will drop to D′, which is identical with D. If the fresh medium added contains the same amount of nitrogen as that contained in the whole algal suspension removed, the nitrogen content of the cells at D′ will reach that of the cells at D in a short period, since the nitrogen newly supplied to highly nitrogen-deficient cells is absorbed very rapidly by the cells, especially in the light. Therefore, the growth curve after the point D′ is expected to show the curve D′C′ which is similar to curve DC, if any special stimulation of growth does not occur and differences of both internal and external conditions of the cells between D and D′ are insignificant. The actual growth curve obtained, however, was D′C″, which in-

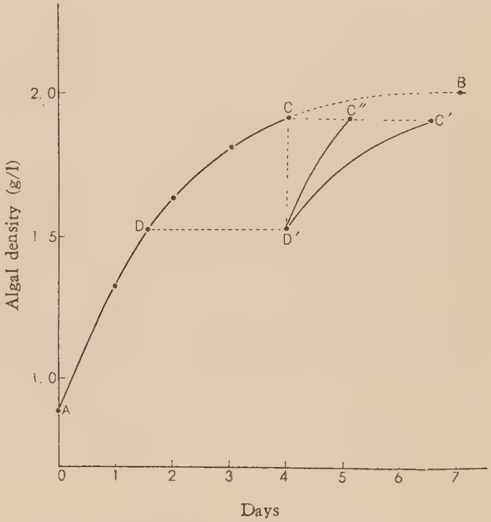

FIG. 6. Stimulation of Growth by N-assimilation in Nitrogen-deficient Cells.

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16) W. E. Fleisher, *J. Gen. Physiol.*, **18**, 573 (1935).

dicates the effect of the stimulation of photosynthesis as described in the previous chapter on net growth.

The above fact indicates, in other words, that the initial algal density C prior to the treatment can be recovered much more promptly than expected from curve DC, and a higher algal density than the maximum density on curve AB can be attained by such treatment.

This stimulation of growth by nitrogen assimilation favours very much the operation of continuous cultivation of fatty cells as will be described in the following chapter.

Results of the semi-continuous and continuous cultivations.

The phenomenon described above is clearly seen in Figs. 7 and 8, which indicate the results of simi-continuous cultivations in different light intensities. In Fig. 7 the cultivation was operated in the light intensity of 30 kilolux. The daily increase of algal density by growth exceeded the daily decrease by harvest, and much higher algal density than in ordinal cultivation seemed to have been attained after a few days' operation. That is, the algal density B is much higher than C which is the density expected when the semi-continuous culture operation were not applied. In Fig. 8, the light intensity was 10 kilolux, and in this case the daily increases and decreases in algal density were incidentally almost the same. In both of the above cases, it is obvious that the actual daily increases in algal density were much larger than those expected when the stimulation had not occurred by nitrogen assimilation.

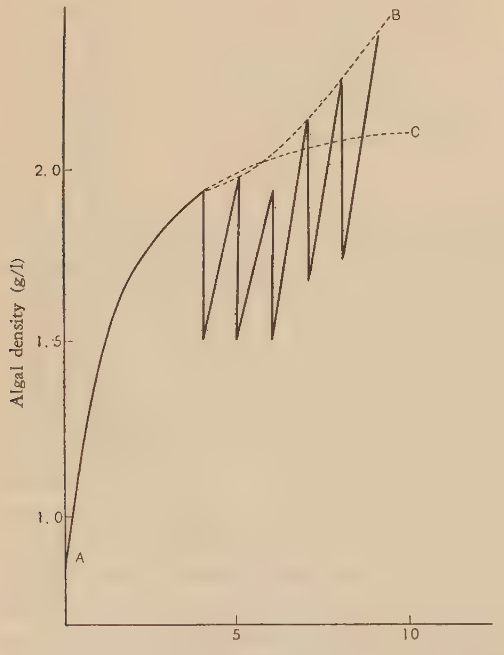

FIG. 7. Semi-continuous Cultivation in 30 killolux.

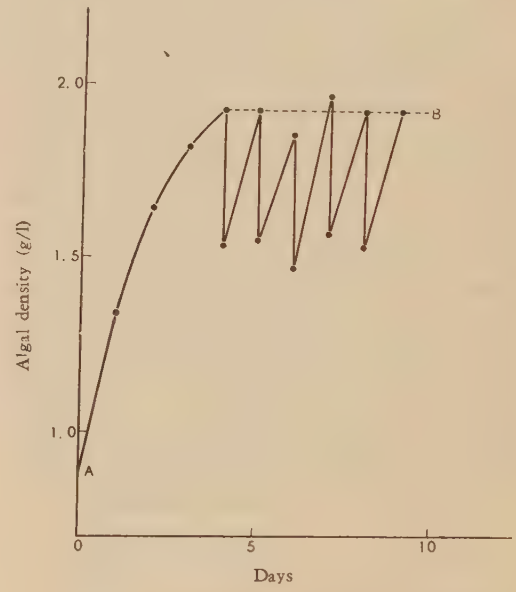

FIG. 8. Semi-continuous Cultivation in 10 kilolux.

Fig. 9 shows the same kind of operation in the light intensity of 30 kilolux. The only difference between Fig. 8 and Fig. 9 is that the semi-continuous culture operation was started at the point where in Fig. 8 the growth curve was still uprising but in Fig. 9 the growth had already stopped. Being different from Fig. 8, Fig. 9 shows a temporary drop in algal density in the early phase of the semi-

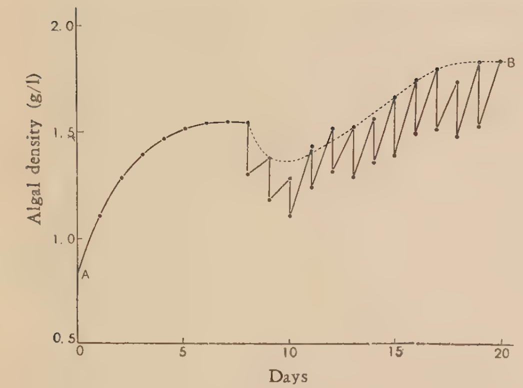

FIG. 9. Semi-continuous Cultivation in 30 kilolux.

continuous operation, and extra growth due to the stimulation was not remarkable during the first few days. In this case, the history of the cells that had not shown any growth for a few days before the operation was started, obviously affected the degree of formation of the stimulation factor.

The tact that the factor is not chlorophyll is clear also in the above experiments. As shown in Table IV, the total amount of chlorophyll contained in a unit volume of the culture distinctly decreased in the course of continuous culture operation in the light intensity of 15 kilolux, while the daily peaks of algal density were maintained almost constant during the operation. In the case of 10 kilolux, chlorophyll increased during the operation, but the algal density was also maintained almost constant. This indicates again that the factor is not chlorophyll. Using the leaves of Sinapis alba, Müller and Larsen[17] observed that the rate of photosynthesis was affected by the nitrogen content of the leaves and not by the chlorophyll content.

TABLE IV. CHANGES OF CHLOROPHYLL AND LIPID CONTENT OF N-DEFICIENT CELLS IN THE COURSES OF CONTINUOUS AND SEMI-CONTINUOUS CULTIVATIONS.

		Chlorophyll content		Algal density	Nitrogen content	Lipid content
		mg/l*	%**	g/l	%**	%**
15 Kilolux, continuous	Start	15.00	0.77	1.95	4.10	40.2
	3rd day	12.00	0.70	1.71	4.62	34.8
	12th day	7.50	0.36	2.12	3.74	36.8
10 kilolux, semi-continuous	Start	14.70	0.76	1.94	4.47	37.3
	3rd day	18.30	0.95	1.92	4.52	36.8
	12th day	18.80	0.93	2.03	4.25	30.2

* mg/cells in 1 litre of suspension.
** percentage in dry weight of cells.

The nature of the factor is not clear, but it might be some individual enzymes or enzyme systems involved in the mechanism of photosynthesis. The fact that the rate of respiration per unit amount of cell nitrogen increases considerably in the course of nitrogen-deficient growth[18], suggests that the total amount of the enzymes related to respiration might possiblly increase, while that of the enzymes related to photosynthesis might possiblly decrease during the nitrogen-free growth.

A continuous culture which was successfully operated in 15 kilolux with changing flow rates between 0.14 and 0.20 is shown in Fig. 10. The algal concentration was maintained at about 2 gram per litre with small deviations corresponding to the flow rates. The culture wes operated for ten days, and it became certain that the continuous cultivation can be operated for a certain length of period.

The yield of fat by semi-continuous or continuous cultivation of nitrogen-deficient cells.

The photosynthetic quotient ($\Delta O_2/-\Delta CO_2$) was thus far reported by many workers to be very close to unity in a very wide range of plants and under various conditions. These

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17) D. Müller and P. Larsen, *Planta*, **23**, 501 (1935).
18) H. Iwamoto and A. Suzuki, This Bulletin, **22**, 420 (1958)

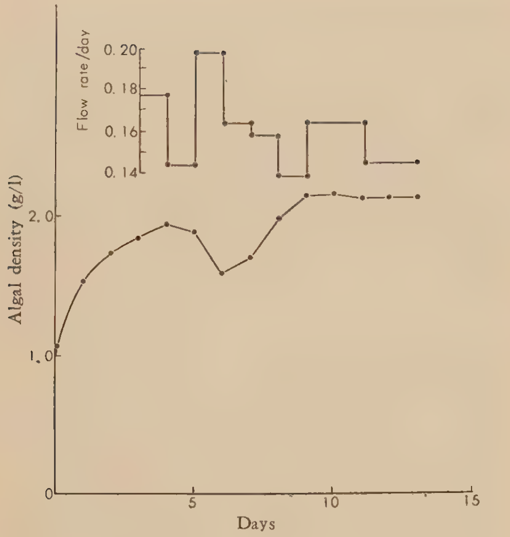

FIG. 10. Continuous Cultivation in 15 Kilolux.

determinations of photosynthetic quotient have generally been made with materials taken from growing cultures. But using the material which had been growing under the nitrogen-deficient condition, the author found higher values of P.Q. remarkably deviated from unity, which indicates that a considerable amount of reducing power produced by light energy absorbed is used in reducing carbon dioxide up to more reduced substances than carbohydrate such as fatty acids[18]. In fact, the P.Q. observed in the experiment shown in Fig. 5 was 1.3 before the addition of ammonium sulphate. After the addition, however, the P.Q. dropped considerably, while the assimilation of nitrogen was proceeding, and then showed a tendency of gradual increase without completely recovering in a short period. The sudden drop of P.Q. seems to be due to protein synthesis.

If it is assumed that, as mentioned previously, the stimulation of photosynthesis occurs not through the activation of the reducing power formation systems but through the activation of the carbon dioxide fixing systems which spend the reducing power, the photosynthetic products might be less reduced after the stimulation occurred than before. This seems likely, and suggests that the lipid content of the cells might decrease to some extent as the continuous culture progresses. This phenomenon seems to occur, in fact. The decreasing tendency of lipid content can be seen in Table IV. The crop obtained on the 12 th day in the continuous (or semi-continuous) operation had a somewhat lower lipid content than the crop obtained at the start of the operation, while the nitrogen-deficiency was a little more advanced in the latter than in the former.

In Fig. 11, lipid content was plotted against nitrogen content in each crop obtained from several series of semi-continuous and continuous cultivations. The marks of the same shape represent a single series. The full mark in eack series represents the first crop which was obtained at the start of each operation. The dotted line indicates the N/Lipid correlation curve of crops obtained by batch cultures[1]. Speaking of cells of the same nitrogen content, cells of more reduced state will appear above this line, and cells of less reduced state will appear below this line. Fig. 11 shows the

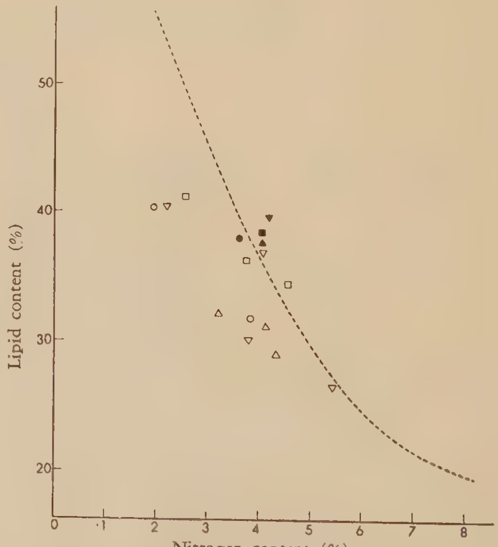

FIG. 11. Lipid Content of the Cells obtained by Continuous and Semi-continuous Cultivations.

crops except the first harvests mostly appeared below this line, and this tendency became conspicuous especially in the more advanced state of nitrogen-deficiency than the initial one. Generally speaking, however, it may be said that this tendency is not very remarkable but rather slight.

As already mentioned, if it were not for the extra growth due to the activation by nitrogen assimilation, the flow rate or the rate of removal per day should be by far smaller than 0.2, in order to maintain the algal density constant in Figs. 8 and 10. And so, it is certain that the yield of fat is much larger thah expected, owing to this kind of stimulatory effect of nitrogen assimulation. It may be quite intersting to investigate what will result when fat yeast is cultivated continuously in nitrogen-deficient state.

It became clear by rough calculation that the yiely of fat in semi-continuous or continuous cultivations is not smaller or perhaps larger than that in batch culture, using the data obtained in the experiments shown in Figs. 9 and 10 and others.

Considering the above successful data, it is expected that the intermittent partial harvest method will be adopted in the future practical out-door cultivation of fat algae, as well as in the case of growing normal cells in a large scale which is at present in practice at the Micro-algae Research Institute of Japan located on the outskirts of Tokyo. According to the senior author[19], nitrogen-free cultivations of Chlorella ellipsoidea were successfully pursued with a small scale out-door culture pond of 2 metre in diameter. The nitrogen-free condition favoured the prevention of contamination trouble. The practical data of outdoor culture of fat cells by batch and semi-continuous cutivations will be presented later on.

SUMMARY

Assimilation of nitrogen by nitrogen-deficient Chlorella cells was investigated. Assimilation occurred very actively at first in the dark, and almost entirely stopped within a few hours, during which remarkable decreases in free reducing sugar, sucrose and water-soluble polysaccharide in the cells were observed. Most of the nitrogen assimilated was stored as soluble nitrogen. Assimilation of nitrogen was accompanied with the activation of respiration and photosynthesis. Activation of respiration ceased when the nitrogen was completely absorbed, but that of photosynthesis lasted thereafter. The latter resulted in stimulation of growth, and favoured very much the operation of semi-continuous and continuous cultivations of nitrogen-deficient fatty cells. It was ascertained that these cultivations can be continued for a certain length of time with much better yield of fat than expected owing to the activation of growth, though the fat content of cells shows a slightly decreasing trend in the course of the operation in the state of equilibrium. The stimulation of photosynthesis was considered to be due to the activation of the enzymes or the enzyme systems involved in the carbon dioxide fixation mechanism.

Acknowledgement. The authors wish to express their hearty gratitude to Prof. T. Asai and Prof. K. Yamada for their encouragement and advices in accomplishing this work.

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[19] H. Iwamoto, unpublished data.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 6, p. 420～425, 1958]

Fat Synthesis in Unicellular Algae

Part IV. Nocturnal Effect on Fat Accumulation in Chlorella

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Photosynthetic and respiratory quotients of Chlorella in nitrogen-deficient growth was investigated. Both quotients were found to be higher than unity, which indicates that the reducing power used in fat synthesis is supplied by both the light energy absorbed and the energy derived by respiration. It being clarified that respiration takes part in fat synthesis to a certain extent, the effect of respiration in dark periods on fat accumulation was investigated in connection with the practical nocturnal effect.

Alternation of light and dark periods once a day gave a favourable effect on fat accumulation, and a distinct difference was observed in fat content of the final crops between the continuous illumination culture and the intermittent illumination culture. And it became clear that the aerated dark periods are effective on fat accumulation especially in the early phase of nitrogen-deficiency.

It was already reported[1] that Chlorella cells continue to fix carbon dioxide actively, when transferred into the nitrogen-free medium, at least in the early phase of nitrogen-deficiency of the cells. In the course of growth in that condition, the cells synthesize and accumulate a larger amount of fat than carbohydrate[2]. It is not clear, however, by what mechanism fat is synthesized in Chlorella and whether fat is a primary product of photosynthesis or a secondary one synthesized from carbohydrate in the process of respiration. In other words, it is uncertain if fat is synthesized by the reducing power directly originated in the light energy or by the reducing power released from carbohydrates by respiration. The same question was presented by Barker[3] in diatoms which were thought to store oils rather than carbohydrate as reserve materials. Whether diatom oils arize as primary products of photosynthesis as suggested by Beijerinck[4] and by Bond[5] or they are secondary products of metabolism, as is true in many other organisms, was studied by Barker with the medium of photosynthetic and respiratory quotients. And he found the photosynthetic quotient ($\Delta O_2/-\Delta CO_2$) to be not larger than 1.05, and concluded that fat production on diatoms is a secondary reaction, not directly associated with photosynthesis. It does not always appear to be so, hawever, since it is possible that the diatom cells were not in the state where active fat synthesis was taking place, because it is likely that the cells transferred from a growing culture into a nitrogen-deficient medium are not in a condition for the immediate rapid synthesis of lipid.

Several workers examined photosynthetic quotients in a wide range of plants and found them to be very close to unity. These determinations have generally been made with materials taken from growing cultures and it is also uncertain whether they were actually in a fat synthesizing state. Among these workers, however, Kok⁶⁾ and Myers⁷⁾ reported 1.09 for P. Q. of Chlorella, Cramer et al⁸⁾ 1.085 for that of Euglena, and Frenkel et al⁹⁾ 1.08 for that of Synechococcus. These values appear to be significantly higher than unity, indicating that a small proportion of fat is produced.

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[1] H. Iwamoto, G. Yonekawa and T. Asai, This Bulletin, **19**, 240 (1955).

[2] H. Iwamoto and N. Nagahashi, *ibid*, **19**, 247 (1955).

[3] H. A. Barker, *Arch. f. Microbiol.*, **6**, 141 (1935).

[4] M. W. Beijerinck, *Rec. trav. bot. neel.*, **1**, 28 (1904).

[5] R. M. Bond, *Bull. Bingham Oceanographic Collection*, **4**, Art. 4, 1 (1932).

But it must be noted that most of these values involve the influence of respiration, and if the respiratory quotients were very high, the true photosynthetic quotients are supposed to have been lower than these values.

The authors determined the photosynthetic and respiratory quotients of Chlorella cells taken from the culture growing in the state of nitrogen deficiency in which active fat synthesis was undoubtedly taking place, and reached to the conclusion that both photosynthesis and respiration are responsible for fat formation.

It is convincing, therefore, that in the practical outdoor culture of nitrogen-deficient cells, the reducing power for fat synthesis is supplied by both processes in the daytime, and at night fat formation does take place through the process of respiration. In the latter case, the principle of fat formation is considered to be the same as that in heterotrophic microorganisms such as fat yeast. Fat accumulation in those microorganisms requires an ample oxygen supply, and therefore, it is conceivable that aeration during the night might have some good effect on fat accumulation in nitrogen-deficient Chlorella cells.

This so-to-speak nocturnal effect was investigated in this study by alternation light and dark periods once a day in the whole course of cultivation.

EXPERIMENTAL METHODS

The organism, the culture apparatus and the medium were the same as described in the previous paper⁽¹⁾. Determinations of nitrogen content, lipid content and dry weight of cells were carried out in the same ways as described in that paper. Warburg's indirect method and direct method were applied to the determination of photosynthetic quotient and respiratory quotient respiratory

RESULTS AND DISCUSSION

The respiratory and photosynthetic quotients in the course of nitrogen-deficient culture are shown in Table I. The quotients were determined with algal suspensions taken from a culture growing in the nitrogen-free medium once a day. Photosynthetic quotients were calculated by correcting the net gas exchange in the light for the gas exchange of respiration determined in parallel dark experiments on the assurance that the rate of respiration and the respiratory quotient are not affected by photosynthesis¹⁰⁾.

The initial algal density at the start of cultivation was 1.37 g/l and the incident light intensity was 5,000 lux throughout the cultivation. The culture was diluted twice to half of the algal concentration in the course of cultivation in order to make the culture progress further in nitrogen-deficiency.

The data shows that both quotients are strikingly higher than unity. The rate of respiration per unit weight of cells and that per unit weight of cell nitrogen are shown in the same table. It is interesting that the former (Qo_2) does not show a marked decreasing trend in spite of the extreme decrease in the nitrogen content of the cells, and therefore, the latter ($Qo_2(N)$) shows a remarkable increase along with the progress of nitrogen deficiency of the cells. Table I shows the increase is especially conspicuous at the later phase of nitrogen deficiency. $Qo_2(N)$ at the later phase is three to four times larger than

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6) B. Kok, *Enzymologia*, **13**, 1 (1938).

7) T. Myers, in "Photosynthesis in Plants", p. 349, Iowa State College Press (1949).

8) M. Cramer and T. Myers, *Amer. J. Bot.*, **37**, 677 (1950).

9) A. Frenkel, H. Gaffron and E. H. Battley, *Biol. Bull.*, **99**, 157 (1950).

10) A. H. Brown, *Amer. J. Bot.*, **40**, 719 (1953).

Table I. The Photosynthetic and Respiratory Quotients and the Rate of Respiration in Nitrogen-deficient Culture

Culture time (hours)	0	24	48	72	168	240	336
Algal density (g/l)	1.37	2.30	2.60	2.88	4.32*	6.32*	7.28*
Nitrogen content(%)	8.50	5.02	4.45	3.85	2.65	1.81	1.54
P.Q.($\Delta O_2/-\Delta CO_2$)	1.18	1.39	1.19	1.28	—	—	—
R.Q.($\Delta CO_2/-\Delta O_2$)	1.16	1.20	1.13	1.31	1.27	–	1.12
Qo_2(μl/mg, hr)	3.2	3.7	2.3	2.0	3.8	2.2	1.9
Qo_2(N)(μl/mg(N), hr)	37.7	75.0	52.2	52.0	143.5	121.5	120.3

* Corrected for the dilutions.

the initial one.

It is very likely that the enzymic equipments of the cells are reorganized during the nitrogen-deficient growth, and their amount for photosynthesis decreases as mentioned in the previous paper[11], and on the contrary, their amount for respiration increases. This hypothesis also receives support from the finding by Virtanen[12] that different nitrogen fractions, including enzymes, in microorganisms are affected to different extents by nitrogen starvation.

Let us make rough calculation on the balance of photosynthetic and respiratory fat formation, based upon these data.

Assuming that the mean value of the photosynthetic quotients was 1.2 during the first two days, and the photosynthetic products were carbohydrate and glycerol trioleinate ($C_{57}H_{104}O_6$)*, the following equation will be given for the photosynthetic assimilation.

$$138\ CO_2+132\ H_2O=1.2\ C_{57}H_{104}O_6+11.6\ C_6H_{12}O_6+(1.2\times 138)\ O_2$$

This equation implies that about twice as much carbohydrate as fat is synthesized by photosynthesis.

The data, on the other hand, shows that 1.37 g of normal cells (Nitrogen: 8.50%, Lipid: 15%, Carbohydrate: 27%) grew up to 2.60 g of nitrogen-deficient cells (Nitrogen: 4.45%, Lipid: 34%, Carbohydrate: 32%) in 48 hrs, synthesizing and accumulating about 0.34 g of carbohydrate and 0.50 g of fat par 85 mg of cell nitrogen as net products of photosynthesis and respiration. When calculation is made on fat formation by respiratory process, applying the above values and equation, on the assumption that Qo_2(N) was 50 throughout that period, the following equation is given for the respiration.

$$30\ C_6H_{12}O_6+100\ O_2=C_{57}H_{104}O_6+123\ CO_2+128\ H_2O$$

These equations imply that about 420 mg of fat and 830 mg of carbohydrate are synthesized by photosynthesis, and about 80 mg of fat is formed by respiration at the respiratory quotient of 1.23 at the expense of about 490 mg of carbohydrate, and consequently, about 500 mg of fat and 340 mg of carbohydrate are accumulated per 85 mg of cell nitrogen in a period of 52 hours. These values well correspond with the values obtained by the experiment, except that the period is a little longer and the respiratory quotient is a little higher than than the observed value, though these deviations may be considered to be rather natural.

On the other hand, if it were not for photosynthetic fat formation at all, the equation for the respiration must be assumed as follows;

$$16\ C_6H_{12}O_6+16\ O_2=C_{57}H_{104}O_6+39\ CO_2+44\ H_2O$$

This implies that about 0.5 g of fat is synthesized by respiration in 52 hours at the respiratory quotient of 2.44, consuming about 1.63 g of carbohydrate synthesized by photosynthesis. But such a high R.Q. value as 2.44 is quite unreasonable or impossible energetically and was never observed.

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[11] H. Iwamoto and H. Sugimoto, This Bulletin, **22**, 410 (1958).

[12] A. I. Virtanen and J. K. Miettinen, *Acta chem. scand.*, **3**, 14317 (1949).

* The fat of chlorella in an advanced stage of nitrogen deficiency is represented by glycerol trioleinate according to the author's unpublished data on the nature of Chlorella fat.

From the above calculation, therefore, it may be concluded that a considerable amount of the reducing power produced by light energy conversion may be utilized for fat synthesis, and that it is photosynthesis that is responsible for the major part of the fat accumulated at least in the earlier phase of nitrogen deficiency. About one sixth of the fat is considered to have been synthesized by respiration in the above case. And it is quite possible that $TPNH_2$ formed in the chloroplasts by the light energy transfer system diffuses out of the chloroplasts and is used as reducing power for fatty acid synthesis, especially in the case where the rate of carbon dioxide fixation is mainly restricted by the decrease in the amount of the related individual enzymes or enzyme systems in nitrogen deficiency as mentioned in the previous paper[11)].

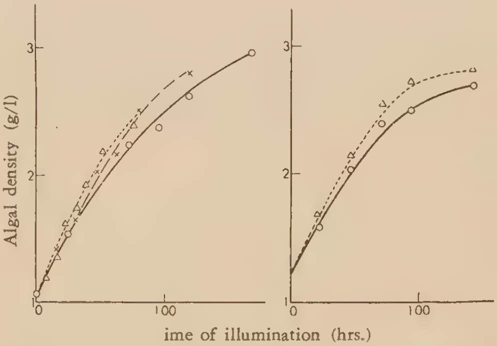
It has long been accepted that the reduction of nitrate which depends upon the photosynthetic activity occurs in illuminated Chlorella and green tissues, when they are supplied with nitrate, though, on the other hand, the reduction of nitrate also occurs in the dark. But reduction of other substances from carbohydrate level to fatty acid level, which depends upon the transformed light energy, has never been ascertained to occur actually. Discussion here strongly suggests its possibility.

There is also a general opinion that $TPNH_2$ does not diffuse out of chloroplasts. But to the authors this seems to occur in a special case such as nitrogen deficiency.

It being clarified that respiration is also responsible for fat synthesis, though to a rather small extent, it is supposed that fat synthesis will proceed by respiration when photosynthesis does not occur in the dark. And so, if the nitrogen-deficient culture is subjected to light and dark alternation, the cells are supposed to accumulate more fat than the cells grown under continuous illumination, compared at the same nitrogen content of the cells. This problem has a practical significance in the outdoor cultivation of fat cells.

To clarify the extent of the effect of respiration in the night, some experiments were conducted, inserting 8 or 16 hours of dark period once a day in nitrogen-deficient cultivation.

The growth curves are shown Figs. 1 and 2. The algal density was plotted against the illumination time length. It is seen that the dark periods affected the growth rather favourably. The nitrogen and fat contents of the final crops of these cultures are shown in Table II. Marked differences are seen in fat content between the light and dark alternation culture and the continuous illumination culture, while the nitrogen contents are almost the same in each series of experiment, and the effect of fat synthesis in the dark periods is clearly observed. This is also illustrated in Fig. 3, in



FIGS. 1 & 2. Growth Curves in Intermittent and Continuous Illumination Cultures.

Dark time per day; 0 hr.: ○——○
8 hrs.: ×---×
16 hrs.: △....△

TABLE II. NOCTURNAL EFFECT ON FAT ACCUMULATION

Dark period in a day (hrs.)	Total light period (hrs.)	Total dark period (hrs.)	Nitrogen content (%)	Lipid content (%)
0	168	0	2.58	50.7
8	256	128	2.50	61.2
16	128	256	2.48	60.5
0	144	0	3.11	44.9
16	144	288	3.09	52.3

which the curve represents the N-Lipid correlation usually obtained by continuous illumination cultures[1]. The cells obtained by the intermittent illumination cultures appear above the curve, which indicates a more reduced state of the cells.

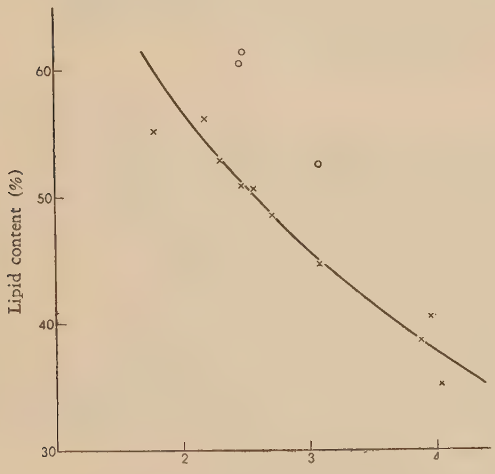


FIG. 3. Lipid Content of the Cells obtained by Intermittent and Continuous Illumination Cultures.
○ Intermittent illumination.
× Continuous illumination.

The effect of dark respiration on fat accumulation is not remarkable or rather negative in the later phase of nitrogen-deficient growth. When cells were put in the dark with aeration for several days, taken out of the light in the actively growing phase of nitrogen-deficient culture, the total amount of fat showed a distinguishable increase, but when cells were taken out in the stationary or slightly growing phase, it did not show any increase or rather showed a little decrease. In Table III, cells were taken in the growing phase in experiments A and A′, and at the beginning of the stationary phase in experiment B. It is conceivable that active photosynthesis at the early phase of nitrogen deficiency can supply a excess of carbohydrate which is convertible into fat by dark respiration, but in the later phase of nitrogen deficiency, where photosynthesis is very faint, convertible carbohydrate is converted into fat by respiration in the light as soon as it is produced by photosynthesis. It is not clear, at present, how the accumulated carbohydrate is converted into fat in the light in the very late phase of nitrogen deficiency, as was observed at the very early stage of this series of investigation. Anyway, it is certain that the accumulated carbohydrate cannot be converted into fat in a large quantity through respiration in the dark.

SUMMARY

Participation degree of photosynthesis and respiration in fat synthesis in Chlorella in nitrogen-deficient growth was investigated with the medium of photosynthetic and respiratory quotients. It became clear that photosynthesis is mainly responsible for fat formation at least in the early stage of nitrogen deficiency and the reducing power resulting from light

TABLE III. EFFECT OF LONG AEROBIC INCUBATION IN THE DARK ON FAT ACCUMULATION.

Experiment	Light period (hrs.)	Dark period. (hrs).	Temp. (dark)	Algal density. (g/l)	Nitrogen content. (%)	Lipid content. (%)
A	46	0	—	1.77	3.89	38.7
	46	96	8	1.55	—	40.1
A′	47	0	—	2.08	—	35.0
	47	96	8	1.96	4.48	37.0
	47	96	25	1.92	4.65	39.2
B	163	0	—	2.30	2.73	48.6
	163	96	8	2.26	2.82	48.3
	163	96	25	2.16	2.92	48.7

energy can be directly utilized for fat synthesis together with that released from carbohydrate through respiration.

The rate of respiration per unit weight of cell nitrogen showed a remarkable increase with the advance of nitrogen deficiency. This fact strongly suggests that reorganization of nitrogen compounds occurs during the nitrogen-deficient growth.

The effect of dark respiration on the accumulation degree of fat was investigated in relation to the practical nocturnal effect in the outdoor culture for fat production. It was clarified that dark periods give a favourable effect on both growth and fat formation, especially in the growing stage. But it became clear that the amount of fat cannot be increased in a large quantity by converting the accumulated carbohydrate through respiration in a prolonged dark period.

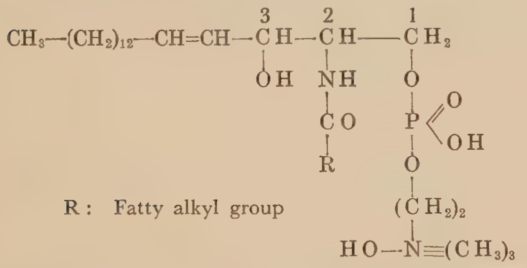
Acknowledgement. The authors wish to express their thanks to Prof. T. Asai and Prof. K. Yamada for their encouragement and advice in accomplishing this work.

Studies on the Conjugated Lipids

Part XIII. Configuration of Sphingomyelin

Sir:

The chemical structure of sphingomyelin has recently been established by Fujino[1] and by Rouser et al.[2] as follows:



$$CH_3-(CH_2)_{12}-CH=CH-\overset{3}{C}H(OH)-\overset{2}{C}H(NH-CO-R)-\overset{1}{C}H_2-O-P(=O)(OH)-O-(CH_2)_2-N(HO)\equiv(CH_3)_3$$

R: Fatty alkyl group

In this structural formula, the configurational establishment is concerned with the two asymmetric carbon atoms and the double bond of the sphingosine moiety. Since it has been verified that the amino carbon atom has the D configuration[3] and the double bond has the *trans* configuration[4], the pending point is that whether the configuration of carbon atom number three is *erythro* or *threo*.

In solving this problem, it seemed pertinent to investigate the configuration of sphingosine, which is usually obtained from acid hydrolysis of sphingolipide. The procedure, however, gives a mixture of the *erythro* and the *threo* bases[5,6], probably because inversion of the configuration occurs in the course of hydrolysis, owing either to the activity of an allylic hydroxyl group at the second asymmetric centre in the molecule[6,7,8], or to the N→O migration of the acyl group in such an amino alcohol compound as sphingolipide[7,9,10]. The author previously reported, in the meantime, a hydrolytic procedure[7,10] which gave no stereoisomerisation at carbon atom number three in the molecule of cerebroside, a kind of sphingolipide. Thus it was decided to apply the same procedure for hydrolysis of sphingomyelin to obtain only one isomer of sphingosine.

A pure preparation of sphingomyelin[11], prepared from crude sphingolipide of beef brain, was hydrolyzed with a saturated solution of barium hydroxide in dioxane–water (1 : 1) to give sphingosine–phosphorylcholine (sphingosine phosphate in part). The product was hydrogenated and the saturated material was subjected to hydrolysis with 1.6 N ethanolic solution of hydrochloric acid. Dihydrosphingosine was obtained in a high yield (over 90%) from the hydrolysate. The triacetyl and tribenzoyl derivatives of the compound were characterized and compared with those of the synthetic bases[12]. Thus, the natural dihydrosphingosine was proved to be

TABLE I

Dihydrosphingosine	Triacetyl– mp. °C	Tribenzoyl– mp. °C
Synthetic *erythro*–	98—100	144—145
Synthetic *threo*–	46	— (only dibenzoyl)
Natural, obtained from sphingomyelin	100—101	145—146

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[1] Y. Fujino, *J. Biochem.*, **39**, 45 (1952).

[2] G. Rouser, J.F. Berry, G. Marinetti and E. Stotz, *J. Am. Chem. Soc.*, **75**, 310 (1953).

[3] H.E. Carter and C.G. Humiston, *J. Biol. Chem.*, **191**, 727 (1951).

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[6] H.E. Carter, O. Nalvandov and P.A. Tavormina, *J. Biol. Chem.*, **192**, 197 (1951).

[7] Y. Fujino and H.E. Carter, Proceedings of the IInd International Conference on Biochemical Problems of Lipids, p. 115, Butterworths Scientific Publications, London (1955).

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[9] L.H. Welsh, *J. Am. Chem. Soc.*, **71**, 3500 (1949).

[10] H.E. Carter and Y. Fujino, *J. Biol. Chem.*, **221**, 879 (1956).

[11] Y. Fujino and T. Negishi, Unpublished.

[12] H.E. Carter, D. Shapiro and J.B. Harrison, *J. Am. Chem. Soc.*, **75**, 1007 (1953).

identical with the synthetic *erythro* base as shown in Table I. No *threo* isomer could be found in the hydrolysis product. These data establish that sphingomyelin has the *erythro* configuration in the sphingosine moiety of the molecule, as in the case of cerebroside[7,10].

The author wishes to express his sincere thanks to Prof. Y. Nakamura and Prof. Y. Obata of Hokkaido University for their kind advice and revisions.

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The Absolute Configuration of Naturally Derived Pyrethrolone and Cinerolone

Sir:

A sole remaining important problem in the field of pyrethrum chemistry is the elucidation of the absolute configuration of naturally derived pyrethrolone and cinerolone.

However, hitherto little is known concerning the stereochemistry of the hydroxyl group of these *cyclo*pentenolones although an extreme ease of racemization has been observed and the fact that both alcohols are alike in dextrorotation suggests that they belong to the same stereochemical series.

As it has been shown in the preliminary study[1], recemic allethrolonemethylether afforded α-methoxysuccinic acid in the sequel of oxidative degradations. In a like manner the dextrorotatory methylethers of naturally derived pyrethrolone and cinerolone were subjected to stepwise oxidations. Thus, (+)-pyrethrolonemethylether (II-a), b.p. 95-6°/1.5 mm., $[\alpha]^{26}_{D}+10.5°$, was at first ozonised to yield, in addition to neutral products, a complex mixture of acid having a laevorotation of $[\alpha]_{D}$ ca. −5°. After the removal of volatile and ether-insoluble acids, the optically active acid fraction mainly consisted of the keto-acid of the structure (III), which, though not isolated pure, was shown by the positive reactions against carbonyl reagents as well as by the formation of bromoform in the subsequent hypobromite oxidation. The potassium hypobromite oxidation of the crude keto-acid gave (−)-α-methoxysuccinic acid (V), m.p. 89-91°, $[\alpha]^{27}_{D}$ −25.0°, (*Anal.* Found. C, 40.73; H, 5.61; Calcd. for $C_5H_8O_5$, C, 40.54; H, 5.44), equivalent weight by titration Found, 73.80 Calcd. for $C_3H_6O(CO_2H)_2$, 74.05. Judging from the manner of consumption of hypobromite, it seems likely that the oxidation proceeds through the intermediate (−)-α-methoxy-α′-ketoglutaric acid (IV).

(+)-Cinerolone-methylether (II-b), b.p. 91-2°/1.2 mm., $[\alpha]^{26}_{D}+10.0°$, also afforded (−)-α-methoxysuccinic acid (V), m.p. 89-92°, $[\alpha]^{27}_{D}-24.6°$, in exactly the same sequel of oxidations.

It is recorded in the literature[2] that optically pure laevorotatory α-methoxysuccinic acid melts at 88-9° and has a rotation −58.18° in acetone at 13°. In spite of the slight discrepancies of these physical properties the observed between the present authors' samples and those in the literature, which might be ascribed to a partial racemization during the processes, the identity was evidenced by analyses and equivalent weight which were in agreement with the theoretical, as well as by infra-red spectrum indicating the necessary absorptions of its functions. Not to mention, it could be nothing else but α-methoxysuc-

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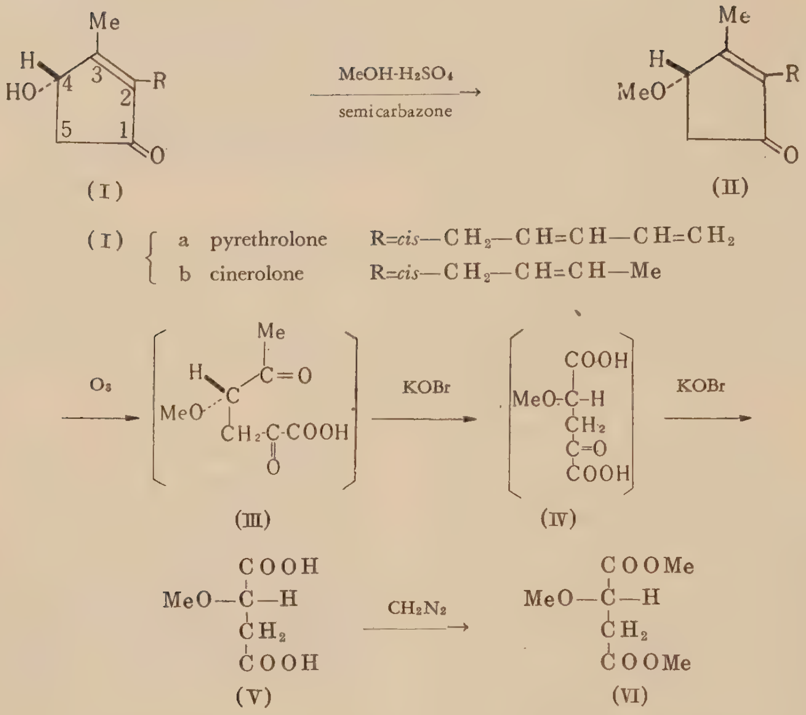
[1] Y. Katsuda, T. Chikamoto and Y. Inouye, This Bulletin **23**, in press.

[2] Purdie and W. Marshall, *J. Chem. Soc.*, **63**, 218 (1893).



(I) a pyrethrolone R=*cis*—CH_2—CH=CH—CH=CH_2
b cinerolone R=*cis*—CH_2—CH=CH—Me

cinic acid in order, as the dicarboxylic acid having the determined constitution of $C_5H_8O_5$ and containing one methoxy-group was optically active. A further evidence was provided by the complete identity of the infra-red spectrum of the methylester (VI), b.p. 67–9°/5 mm., n_D^{20} 1.4319, $[\alpha]_D^{27}$ $-20.4°$ (from pyrethrolone-methylether), b.p. 67–9°/5 mm., n_D^{20} 1.4301, $[\alpha]_D^{27}$ $-19.8°$ (from cinerolone-methylether), with that of an authentic specimen of the corresponding racemate, prepared by the procedures of Reichstein[3,1].

Since the *cyclo*pentenolone structures of pyrethrolone, cinerolone and consequently, of their methylethers have, beyond doubt been established as (I) and the sole asymmetric carbon atom involved is that of position-4, it is reasonably concluded that the asymmetric carbon atom in the resulting (−)-α-methoxysuccinic acid corresponds to that of position-4 in the parent compounds, the α-carboxylic carbon to $C_{(3)}$ and the β-carboxylic carbon to $C_{(1)}$ respectively.

Since (−)-α-methoxysuccinic acid was related to (−)-malic acid and this, in turn, to glyceraldehyde by Freudenberg[4], the chemical conversion of (+)-pyrethrolone- and (+)-cinerolone-methylethers to (−)-α-methoxysuccinic acid achieves the ultimate correlation between these alcohols and (−)-glyceraldehyde, thereby establishing the absolute configuration of the asymmetric carbon-4 of naturally derived pyrethrolone and cinerolone as Ia and Ib.

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[3] A. Larson and T. Reichstein, *Helv.*, **32**, 2003 (1949).

[4] K. Freudenberg, *Ber.*, **47**, 2027 (1914).

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[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 6 p. 429~431, 1958]

Biochemical Studies on " Bakanae " Fungus. Part 45

Chemical Structure of Gibberellins. Part XII

Sirs:

As it has been described in our previous report[1], the position of the secondary hydroxyl group of gibberellin A_1 (I) was confirmed to be the C–2 of ring A, and the partial structural formulae of A_1 and A_3 were proposed.

The lactone carbonyl has now been elucidated to be located at C–3 of ring A.

First, the relative position of lactone carbonyl with the secondary hydroxyl group was determined in the following way.

The pentaol (II), $C_{19}H_{30}O_5$, m.p. 208–10°, which was obtained by reduction of gibberellin A_1 methyl ester with lithium aluminum hydride, did not consume periodic acid[2], but gave a monoacetonyl monoanhydro compound (III), $C_{22}H_{32}O_4$, m.p. 137–9°, when its acetone solution was treated with sulfuric acid, showing the presence of one 1,3-glycol linkage in its molecule. Moreover, the C–2 keto-compound (IV), $C_{19}H_{24}O_6$, d.p. 240°, (infrared absorption: lactone 1760 cm^{-1}, 6-membered ring ketone 1709 cm^{-1}, carboxyl 1695

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[1] Y. Seta, N. Takahashi, H. Kitamura and Y. Sumiki, This Bulletin, **22**, 62 (1958).

[2] N. Takahashi, Y. Seta, H. Kitamura and Y. Sumiki, This Bulletin, **21**, 327 (1957).

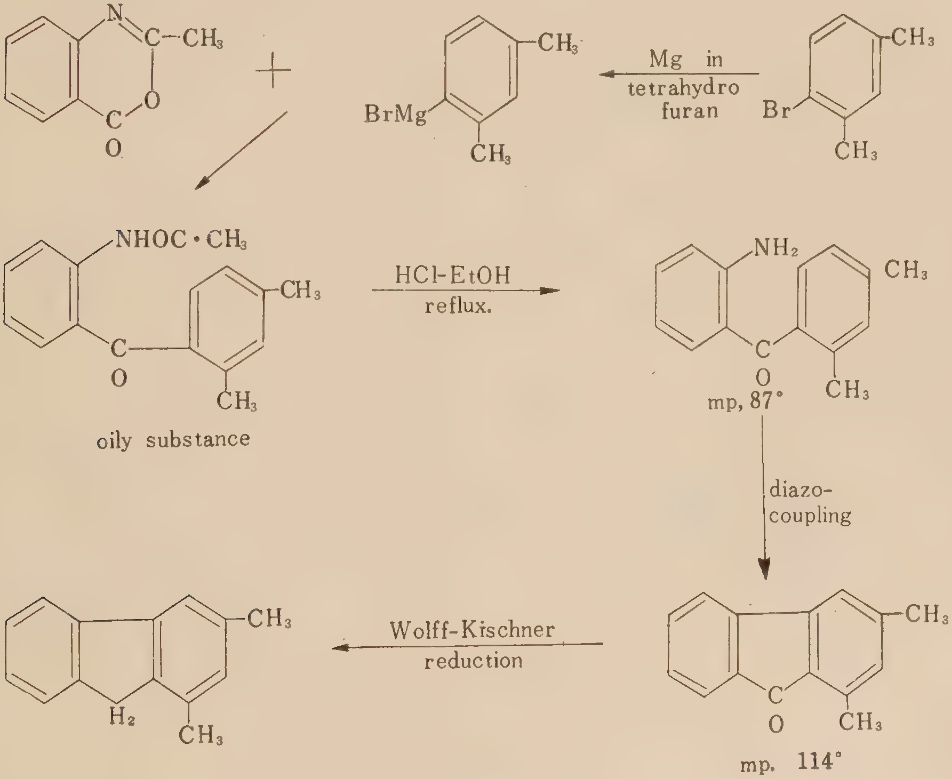

FIG. I

cm^{-1}), obtained from dihydrogibberellin A_1 by oxidising with chromic acid in pyridine solution, gave, when boiled with 25% sulfuric acid, a keto-monocarboxylic acid (V), $C_{18}H_{24}O_4$, d.p. 215–7°, as the main product, losing one mole of carbon dioxide, and an α,β-unsaturated ketone (VI), $C_{17}H_{24}O_2$, m.p. 193°, as the minor product, losing two moles of carbon dioxide. The product (V) showed infrared absorption at 1724 cm^{-1} and 1709 cm^{-1}, indicating the presence of 6-membered ring ketone and carboxyl respectively, and the product (VI) showed infrared absorption at 1642 cm^{-1}, and ultraviolet absorption at 247mμ (ε, 9500) and 310mμ (ε, 137), indicating the presence of α,β-unsaturated ketone. However, the absorption band of lactone at 1760 cm^{-1} that existed in (IV), disappeared completely in both products, (V) and (VI). Since the elimination of lactone could not be observed by the similar treatment of dihydrogibberellin A_1, this fact shows that the ketone at C-2 is located at the β-position towards the lactone carbonyl and facilitates the elimination of lactone. On the other hand, the hydrogenolysis product of gibberellin A_3, (VII), $C_{19}H_{28}O_6$, in which the lactone ring received reductive cleavage and converted into carboxyl[2], gave, when oxidised with chromic acid in its acetic acid solution, a ketomonocarboxylic acid (VIII), $C_{18}H_{26}O_4$, m.p. 198–200°, simultaneously liberating one mole of carbon

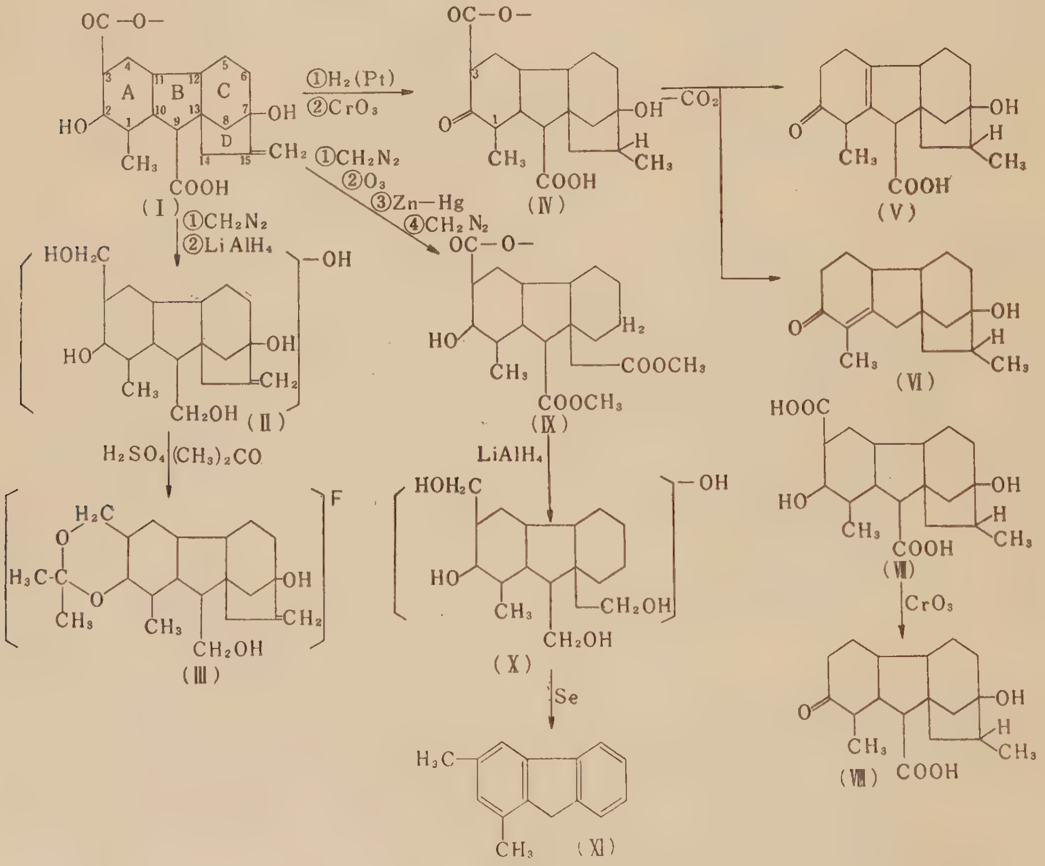

FIG. II

dioxide with oxidation of the secondary hydroxyl.

From these facts, it is ascertained that the lactone carbonyl should be attached to C-3 or C-1 of ring A.

Further, the position of the lactone carbonyl was determined from the following experimental results.

The ozonolysis product, $C_{19}H_{24}O_8$, of gibberellin A_1 methyl ester, gave, when reduced with amalgamated zinc and hydrochloric acid, a desoxo-product, $C_{19}H_{26}O_7$[1]. When this compound was methylated with diazomethane and the methylester, (IX), $C_{20}H_{28}O_7$, m.p. 154–6°, was subsequently boiled with lithium aluminum hydride in its tetrahydrofuran solution, carbomethoxyl and lactone carbonyl groups were reduced to hydroxymethyls, giving a pentaol, (X), $C_{18}H_{32}O_5$, m.p. 217–9°. This pentaol gave, when dehydrogenated with selenium at 360–400°, a crystalline hydrocarbon, m.p. 54–67°, which was considered to be a mixture of 1,3-dimethyl fluorene (ca. 80%) and 1-methyl fluorene (ca. 20%) from the ultraviolet and infrared examination. This hydrocarbon, after recrystallization five times from dil. ethanol, showed m.p. 94–7° and was proved to be identical with 1,3-dimethyl fluorene, m.p. 99–100°, (XI), which was synthesized through the process shown in Fig. I, in mixed m.p., ultraviolet and infrared spectra.

If the hydroxymethyl group, which was derived from lactone carbonyl is attached to C-1 together with original methyl group, it will split off during dehydrogenation to give 1-methyl fluorene only, and 1,3-dimethyl fluorene should not be formed. Therefore, the 3-methyl substituent must originate from the hydroxymethyl at C-3 of pentaol (X), which was formed from lactone carbonyl by lithium aluminum hydride reduction.

From these results above-described, it is concluded that the position of the lactone carbonyl of A_1 must be C-3 of ring A.

As the position of the original carboxyl in A_1 was already determined by Cross et al.[3], who obtained (±) methyl-1,7-dimethyl fluorene-9-carboxylate by dehydrogenation of gibbertricarboxylic acid monomethyl ester, we propose the partial structural formula (I) for gibberellin A_1 as shown in Fig. II and the formulae of each of the products and the reaction procedure discussed above are to be represented as Fig. II-(I～XI).

There is, however, still one more point which requires clarification. When the hydrogenolysis product, (VII), of A_3 was treated with acetic anhydride, an anhydride, $C_{23}H_{30}O_7$, m.p. 165–7° (infrared absorption: anhydride 1801, 1766 cm^{-1}, acetate 1745 cm^{-1}) was obtained. As far as this experiment is concerned, the position of the lactone carbonyl at C-1 seems to be preferable. However it is also considered that the formation of 8-membered anhydride might be possible if the stereochemical conditions were suitable and the infrared absorption of this group might be same as that of the 6-membered one. Therefore the location of lactone carbonyl at C-3 is believed to be correct.

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[3] B.E. Cross et al., *Chem. & Ind.* **1956**, 954.

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 6, p. 432～433, 1958]

Biochemical Studies on "Bakanae" Fungus. Part 46

Chemical Structure of Gibberellins. Part XIII

Sirs:

In the previous paper[1], the attaching position of lactone carbonyl of gibberellins A_1 and A_3 was reported. In this communication, we wish to report on the attaching position of lactone alkyl 0 and to present the final structure of gibberellins A_1 and A_3.

When gibberellin A_3 was treated with bromine in tetrahydrofuran at $-5°\sim0°$, and the reaction product was chromatogramed on silicic acid, monobromogibberellin A_3, $C_{19}H_{21}O_6Br$, d.p. 240–2°, was obtained. Monobromogibberellin A_3 gave the monobromogibberellin A_1 in a 30% yield on catalytic reduction at neutral condition absorbing one mole of hydrogen and also, gave the gibberellin C on debromination with zinc and acetic acid accompanied with a simultaneous reduction. From these results, it is clear that A_3 undergoes the Wagner rearrangement at ring C, D in the same mechanism as that of gibberellin A_1 and the double bond in ring A of gibberellin A_3 remains unaffected in monobromogibberellin A_3. As monobromogibberellin A_3 methyl ester gave an acidic hydrogenolysis product, $C_{20}H_{27}O_6Br$, d.p. 206°, on catalytic hydrogenation by Pt-oxide in a 40% yield, it is confirmed that the double bond of monobromogibberellin A_3 is located at the same position as that of gibberellin A_3. Therefore, the monobromogibberellin A_3 was used to determine the position of the double bond in ring A. Monobromogibberellin A_3 gave monoacetylmonobromogibberellin A_3, $C_{21}H_{23}O_7Br\cdot H_2O$, d.p. 175° by treatment of acetic anhydride and pyridine at room temperature and monoacetylmonobromogibberellin A_3 methyl ester $C_{22}H_{25}O_7Br$, m.p. 178–80°, was obtained by treatment of diazomethane of the acetyl derivative.

When monoacetylmonobromogibberellin A_3 methyl ester was treated with ozone in chloroform solution and ozonide was decomposed by steam distillation, a non-volatile acidic substance was obtained. Although the ozonolysis product could not be purified by various methods into crystalline form, the result of titration of white powder obtained by silicic acid chromatography showed that it is monobasic. This result leads us to conclude that the double bond exists at the position of C-4～C-11. Because, if the double bond exists at the position of C-1～C-10, the oxidation product by ozone should not be a carboxylic acid but diketo compound. Moreover, when the ozonolysis product was boiled with 33% H_2SO_4, evolution of 0.5 mole of CO_2 was observed. This easiness of decarboxylation shows that the monocarboxylic acid is β-oxocarboxylic acid. From these facts we assigned the position of the double bond of monobromogibberellin A_3 and gibberellin A_1 to C-4～C-11. It has also been clarified that alkyl 0 of lactone is allylic to the double bond, because of the easy occurrence of hydrogenolysis on the catalytic hydrogenation of gibberellin A_3 and monobromogibberellin A_3. If we put the double bond at C-4～C-11, alkyl 0 of lactone ring should be attached to C-12 or C-10. From the fact that aromatization of gibberellin A_3 occurs with great ease, the position of C-12 is unlikely. As a result, the structure of gibberellin A_3 is formulated as (I) and those of gibberellin A_1 and monobromogibberellin A_3 as (II) and (III), respectively.

As shown in (I), (II) and (III), the lactone of gibberellin is δ and not γ. In the infrared spectrum of gibberellins A_1 and A_3, the lac-

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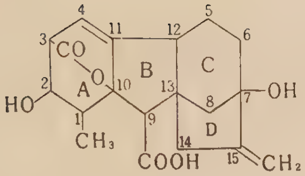
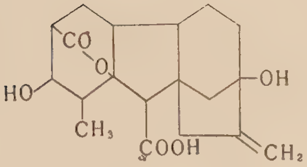
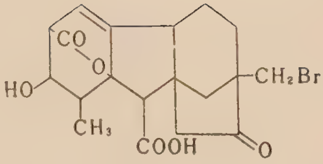
[1] Part VII of this series.



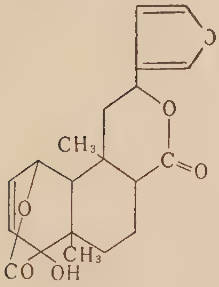
(I)



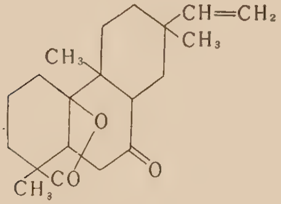
(II)



(III)



(IV)



(V)

tone carbonyl band of A_1 ester appears at 1759 cm^{-1} and that of A_3 ester at 1766 cm^{-1} which are usually thought to be γ-lactone. But if lactone ring exists in ring system, there might be a shift of the carbonyl band to high frequency for the structural strain. In fact, carbonyl band of δ-lactone of columbin (IV)[2] appears at 1750 cm^{-1}, and that of γ-lactone of rosenolactone (V)[3] at 1786 cm^{-1}. From these examples, it is considered reasonable that the absorption band of gibberellins shows the existence of δ-lactone.

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[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 6, p. 434~435, 1958]

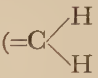
Biochemical Studies on " Bakanae " Fungus. Part 47

Chemical Structure of Gibberellins. Part XIV

Sirs :

As previously reported[1], gibberellin A$_2$ was a lactonic monobasic acid, having the same functional groups, i.e. a secondary and a tertiary hydroxyl, a lactone and a carboxyl and the same skelton as those of gibberellins A$_1$ and A$_3$, except the absence of the ethylenic double bond and high C–Me value. In this communication, we wish to represent the partial structure of A$_2$ and A$_4$ by formulae I and II respectively on the basis of the following results.

Acetylation of A$_2$ gave only one monoacetyl derivative, m.p. 204–5°, i.e. the acetylated compound of its secondary OH, whilst dihydro A$_1$ gave two monoacetyl stereoisomers, m.p. 120–2° ; 202–4° and two diacetyl stereoisomers, m.p. 176–8°; 210–2°. By dehydration with POCl$_3$ in pyridine, both the secondary OH and tertiary OH of A$_2$ were simultaneously dehydrated to give a dianhydro-derivative, $C_{20}H_{24}O_4$, m.p. 145–6°, whilst dihydro A$_1$ gave a monoanhydro-derivative, since only the secondary OH was dehydrated and the tertiary OH could not be dehydrated by trance coplanar elimination on account of steric hindrance. These results elucidate that A$_2$ and dihydro A$_1$ are each other not stereoisomers, and the tertiary OH of the former is located at a different position from that of the latter, although they have the same empirical formulae.

Dehydration with POCl$_3$ in pyridine of the above monoacetyl A$_2$ methylester (I–a) readily gave a dehydrated product, $C_{22}H_{28}O_6$, m.p. 129–130°, (I–b), resulting in the formation of an exocyclic methylene in a five-membered ring by the dehydration of the tertiary OH. This fact is supported from the presence of bands at 1657cm^{-1} (C=C stretching) and 876cm^{-1} ( $=C{<}^{H}_{H}$ bending) in its infrared spectrum, i.e. the relative high frequency[2] of the C=C stretching vibration indicates that the vinylidene group is attached to a slightly strained ring (five-membered ring).

The above results were confirmed from the following experiments. Ozonolysis of the dehydration product (I–b) gave formaldehyde (ca. 50%), formic acid (ca. 30%) and a neutral keto-compound, $C_{21}H_{26}O_7$, m.p. 187–9°, (I–c). The keto-compound showed carbonyl bands at 1750 cm^{-1} and λ_{max} 280 mμ ε=25, which indicate the presence of a carbonyl group in a five-membered ring. Oxidation of the above keto-compound with SeO$_2$ gave a 1,2-diketo-compound, λ_{max} 315 mμ ε=60. These results show the presence of the –CH$_2$–CO– in a five-membered ring, Selenium dehydrogenation of the keto-compound (I–c), afforded 1-methylfluorene. Each reaction product by an excess of methylmagnesium iodide on the above keto-compound (I–c) and on the monoacetyl derivative of A$_2$ (I–a), was identical by infrared spectra comparison. These facts elucidate that the position of the tertiary OH of A$_2$ should be C–15.

Gibberellin A$_4$[3] had an ethylenic double bond and absorbed one mole of hydrogene by catalytic hydrogenation to yield dihydro A$_4$. As ozonolysis of A$_4$ gave formaldehyde and a keto-compound, $C_{19}H_{24}O_6$, m.p. 203°, A$_4$ is considered to have an exocyclic methylene group. Selenium dehydrogenation of A$_4$ gave 1,7-dimethylfluorene, accompanying a small amount of 1-methylfluorene. Monoacetyl A_4 methylester was proved to be identical with the monoacetyl A_2 monoanhydro-derivative, $C_{22}O_{28}O_6$, m.p. 129–130°, (I-b) by the mixed melting point and infrared spectra. Furthermore, A_4 anhydro-derivative was, also, proved to be the same compound with A_2 dianhydro-derivative, $C_{20}H_{24}O_4$, m.p, 145–6°, (II-a) by the mixed melting point and infrared spectra comparison. Therefore, the partial structures of A_2 and A_4 are represented by formulae I and II.

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[2] W. Bottomley et al., *Jour. of Chem. Soc.*, **1955**, 2624.

[3] N. Takahashi, Y. Seta, H. Kitamura and Y. Sumiki, This Bulletin, **21**, 396-8 (1957).

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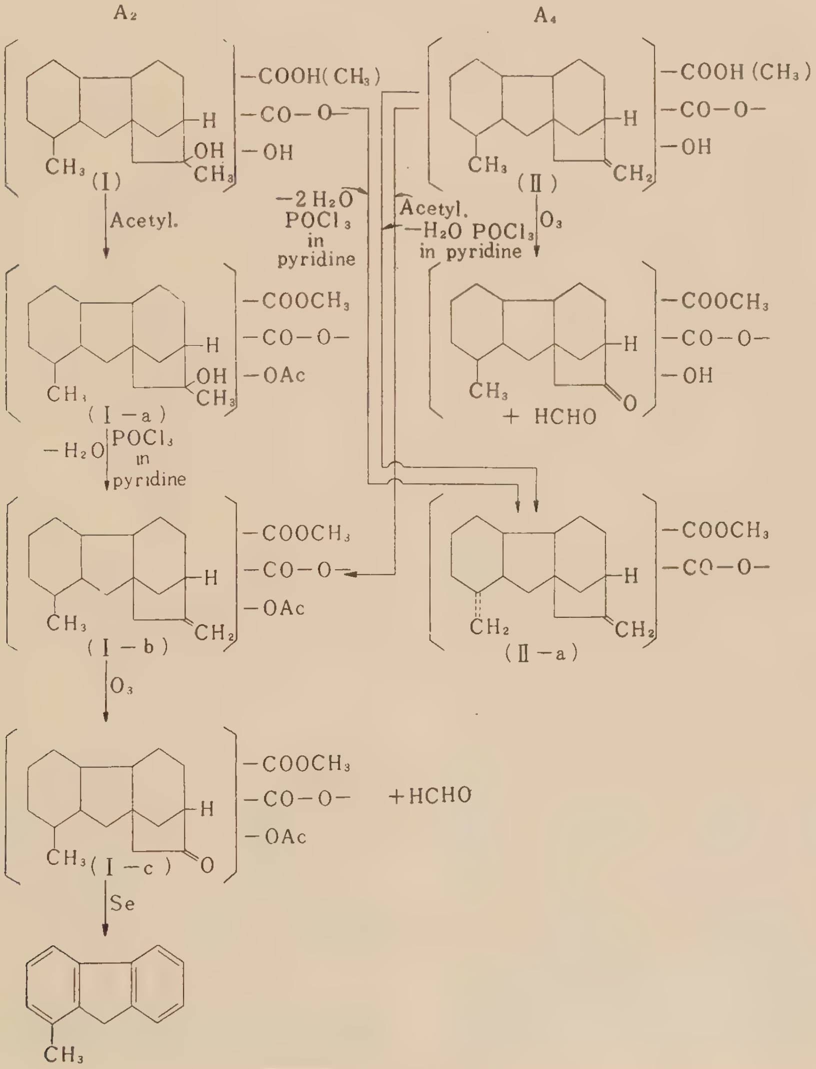

[Bull. Agr. Chem. Soc. Japan, Vol. 22, No. 6, p. 436~437, 1958]

Fucose as a Constituent of Hemicellulose B_1 from Broad-Bean Seeds

Sir:

In 1956 we[1] announced that the hydrolyzate of hemicellulose B_1 prepared from dehulled seeds of the broad bean (*Vicia faba*) consisted of glucose, xylose, galactose, and ribose in approximate molar ratios of 46 : 35 : 14 : 5. The detailed report was published in 1957[2]. Narasaki[3] found that the fraction corresponding to ribose gave negative reactions for pentoses.

The hydrolyzate of broad-bean hemicellulose B_1 obtained by the method reported previously was chromatographed on paper and on a cellulose column to isolate the unidentified sugar as a pure solution.

The color of the spot on paper was not reported previously, since it was very obscure. The colors of the spot showed that the spot corresponded not to pentose but to methylpentose by aniline phthalate (brown), *p*-anisidine hydrochloride (yellow), and vanillin-perchloric acid (orange).

Pure solutions of the unidentified sugar were examined by several spectrophotometric methods in comparison with comparable amounts of xylose, rhamnose, glucose, and fructose. All such experiments showed that the unidentified sugar was methylpentose. By the Dische's special cysteine-sulfuric acid reaction for methylpentose[4] and general cysteine-sulfuric acid reaction[5] the single absorption maximum at 400mμ increased gradually up to 24 hr. By the anthrone reaction[6] rhamnose and fructose were more unstable than glucose in the first several minutes in the absorption at 620mμ; the unidentified sugar behaved like rhamnose. By the Gibbon's thioglycolic acid-sulfuric acid reaction[7] the single absorption maximum at 400mμ for methylpentose was evident in comparison with much lower absorption for glucose and xylose. Finally by the Drury's orcinol reaction[8] xylose had a high peak at 660mμ, while the unidentified sugar had much lower absorption with a low peak at 430mμ.

Paper chromatography showed that this methylpentose was identical with fucose, when compared with the authentic sample of L-fucose*. The R_F values are shown in the table. The paper chromatography was made by ascending technique at rather higher room temperatures (about 25~30°).

	Unidentified sugar	Fucose	Rhamnose	Ribose
BuOH-AcOH	0.42	0.42	0.47	0.40
BuOH-C_5H_5N	0.39	0.39	0.47	0.40
BuOH-EtOH	0.34	0.34	0.40	0.33
PhOH	0.65	0.65	0.61	0.62

The solvent systems were, in the order shown in the table, *n*-butanol-acetic acid-water (4 : 1 : 2), *n*-butanol-pyridine-water (3 : 1 : 1.5, the upper layer), *n*-butanol-ethanol-water (5 : 1 : 4, the upper layer), and phenol-water (4 : 1).

Among the three naturally occurring methylpentoses rhamnose and quinovose differ in its R_F values. Thus we conclude that the fourth constituent is fucose. The erroneous presumption as ribose was due to the ignorance of the color of the spot on the paper. The spot was not red but yellow with *p*-

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2) S. Kawamura, T. Narasaki, R. Kusuhara, and T. Satô, *Tech. Bull. Fac. Agr. Kagawa. Univ.*, **8**, 201 (1957) (in Esperanto).
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7) M.N. Gibbons, *Analyst*, **80**, 268 (1955).
8) H.F. Drury, *Arch. Biochem.*, **19**, 455 (1948).

* A commercial product of Pfanstiehl Chemical Company, Waukegan, Illinois, U.S.A.

anisidine hydrochloride. We presumed that the spot which should be red seemed yellow owing to the scarceness in quantity of the sugar.

Thanks are due to Dr. Yosito Sakurai and Dr. Saburo Funahashi, professors at the University of Tokyo, for their interests in this work. A part of the expenditure was defrayed by the research fund donated by the Ministry of Education to S. K. (A study on ribose as a constituent sugar of hemicelluloses, 1957).

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Studies on Vitamin B_{12}-Synthesizing Enzyme. Part I.

Isolation of an Enzyme Preparation from *Streptomyces olivaceus*.

Sir:

It is widely known that *Streptomyces* produces vitamin B_{12}, and the existence of a synthetic enzyme in the cells has been expected. We have therefore attempted studies on the isolation of the enzyme from *Streptomyces olivaceus* PD 61, and activity of the enzyme preparation was tested for the biosynthesis of vitamin B_{12} in vitro, in a synthetic medium (pH 7.2) containing 140 γ of Cobalt–porphyrin (Na salt), 30 γ of 5,6–dimethylbenzimidazole, 30 γ of D–ribose, 48 γ of DL–threonine, 20 γ of diammoniumhydrogenphosphate, 20 γ of potassium cyanide and 6 γ of ATP per ml.

Streptomyces olivaceus was first cultured in 80–ml of Dulaney medium in the range of 28–32°C for seven days under shaking. Then, the cells were collected by centrifugation and washed with distilled water repeatedly. The cells thus obtained were treated with a Raytheon Sonic Oscillator for thirty minutes, and the sediment (1.49 g) was washed with 15 ml of 10% sodium chloride. The insoluble portion (1.39 g) was subsequently treated with 14 ml of 0.1% sodium hydroxide. To the alkali–insoluble fraction (1.27 g) was added 13 ml of 70% ethylalcohol, and 10 ml of the alcohol–soluble fraction was digested at 37°C with 500 mg of diastase and a few drops of toluene. After incubation for 45 hours, the digest was treated with 90 ml of barbiturate buffer (pH 7.2), and the mixture was centrifuged. The upper–layer was saturated with ammonium sulphate. After treatment for one hour, the precipitate was sedimentated on a centrifuge. The resulting product was suspended in 10 ml of barbiturate buffer, pH 7.2 and dialysed through cellophane membrane against running water for three days. Twenty–five ml of the dialysed solution was freeze–dried over a mixture of dry–ice and acetone, yielding 7 mg of enzyme preparation in ammorphous powder.

The enzyme preparation prepared in this manner was subsequently tested for its vitamin B_{12}–producing activity in vitro by application of the ordinary tube method. Determination was carried out at 28°C using 10 ml of the synthetic medium (pH 7.2) described above each tube containing 2 mg of the enzyme preparation. The amount of vitamin B_{12} pro-

TABLE I

Components of Reaction Medium				Total Amount of Vitamin B_{12} produced, γ per liter	
Enzyme preparation, mg/tube	ATP, γ/tube	KCN, γ/tube	Factors*	Euglena method	Ochromonas method
2	60	200	+	4.86	0.98 (1.92)***
2	—	200	+	—	0.58
2	60	—	+	—	0.56
—	60	200	+	0.52	0.02
—	—	—	—	0	0
2**	60	200	+	0	—

* Factors except ATP and KCN from the complete reaction medium.

** Enzyme preparation inactivated by treatment with alkali (100°, 30 min).

*** This value means the highest one obtained in three tests.

duced in a period of one hour was accurately estimated by microbioassay with both *Euglena gracilis* and *Ochromonas malhamensis*. The results obtained are shown in Table I.

The above results may indicate that the enzyme preparation isolated from *Streptomyces olivaceus* possibly activates the synthesis of vitamin B_{12}. Further studies on the properties of the enzyme are now in progress.

We wish to express our sincere thanks to Prof. S. Funahashi, Prof. K. Iwamoto, Mr. T. Suzuki, Prof. T. Kobayashi, Dr. A. Yoshida, Mr. H. Sakai and Dr. S. Kinoshita for their kind advice. This work was aided in part by a grant from the Scientific Research Fund, Minisrty of Education, to which the authors are indebted.

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Chemical Studies on the Tanning Properties of Animal Skin. Part II. On the Reaction between Skin Collagen and Phenol (-OH) or Benzoic Acid (-COOH) (p. *651~656*)

By Yasushi SATO*

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(1) To make clear the reaction between collagen and gallotannin, some experiments were carried out on the reaction between skin collagen and phenol or benzoic acid which contain either OH group or COOH group that are seemd to be fundamentally functional group of tannin.

(2) Adsorption of phenol is accomplished in 10 minutes. Adsorbed amounts are decreased by addition of NaOH, but not almost by HCl. The correlation between the adsorbed amounts (x) and its equilibrium concentration (C) is fitted for Freundlich's adsorption isotherm ($X=KC^n$) in two parts having different coefficient (n), 0.9 and 2.5 respectively. Adsorbed phenol in acid solution are removed with ease by successive extraction with water, but it becomes more difficult to remove if with the solution having same pH as the solution after reaction. Phenol is adsorbed in organic solvents up to the same amounts as in water. Adsorption of phenol by such soluble protein as casein, coagulated egg albumin and fresh egg white protein are similar to it by skin collagen. Fresh egg white protein precipitated by phenol can not be resolved in water by removing adsorbed phenol. Thinking from above these results, it is estimated that adsorptive reaction between phenol and collagen is both ionic and non-ionic.

(3) Adsorption of benzoic acid is accomplished in one hour. Amounts of adsorbed benzoic acid are decreased by addition of HCl and NaOH. The correlation between the adsorbed amounts and its equilibrium concentration is partly fitted for Freundlich's adsorption isotherm, and in that part coefficient (n) is 0.8. It is more difficult to remove adsorbed benzoic acid than phenol by successive extraction with water. Benzoic acid is not almost adsorbed in organic solvents. Thinking from above these results, it is estimated that adsorptive reaction of benzoic acid is mainly ionic.

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Chemical Studies on the Tanning Properties of Animal Skin. Part III. On the Reaction between Skin Collagen and Salicylic Acid or Sulfosalicylic Acid. (p. *656~661*)

By Yasushi SATO*

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(1) To make clear the reaction between skin collagen and gallotannin, some experiments were carried out on the reaction between skin collagen and salicylic acid or sulfosalicylic acid which contain both OH group and COOH group that are seemed to be the fundamentally functional group of tannin.

(2) Amounts of adsorbed salicylic acid and sulfosalicylic acid are decreased by addition of HCl or NaOH. The correlation between adsorbed amounts (x) and its equilibrium concentration (C) is fitted for Freunnlich's adsorption isotherm ($x=KC^n$) in two parts having different coefficient (n), respectively 0.9 and 0.3 for salicylic acid, respectively 3.4 and 0.25 for sulfosalicylic Acid. It is more difficult to washing out adsorbed salicylic acid than benzoic acid, sulfosalicylic acid than salicylic acid. Salicylic acid is not adsorbed in 95% alcohol solution, but in 50% aceton solution two thirds of adsorbed amounts in water solution is adsorbed. Adsorption of sulfosalicylic acid in these solution is almost same as in water solution. The correlation between adsorbed amounts of sulfosalicylic acid by soluble protein and its equilibrium concentration is similar to that in the case of skin collagen. When it is precipitated completely, adsorbed amounts become constant. Thermal shrinkage temperature of skin is most greatly lowered by the effect of $\left|\begin{array}{l}-\mathrm{OH}\\ -\mathrm{COOH}\end{array}\right.$ group, and more greatly lowered by the effect of HSO_3 group than by the effect of OH group.

(3) Thinking from above these results, it is estimated that adsorptive reaction between skin collagen and salicylic acid is due to the reaction between chelating structure of OH and COOH group in salicylic acid

and NH_2 group and non-dissociated groups of skin protein, and that adsorptive reaction between skin collagen and sulfosalicylic acid are due to the reaction between dissociated acidic group or OH group of sulfosalicylic acid and dissociated cationic group or non-dissociated groups of skin collagen.

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X-ray Diffractometric Studies on Starches. Part III. Disorganization of Potato Starch Granule by Iodine Potassium Iodide Solution. (p. *661~667*)

By Susumu HIZUKURI

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The behaviors of potato starch granule in iodine potassium iodide solutions were examined by a X-ray diffractometer and a photomicroscope. Iodine was adsorbed rapidly into the inner part of the starch granule. The original crystallite structure of the granule was disordered gradually by the increasing amount of adsorbed iodine until the granule showed an amorphous X-ray pattern of starch-iodine complex. When the iodine was removed from the iodine stained granule by the addition of 0.1 N sodium thiosulfate solution, the original X-ray B-pattern recovered partly and birefringence of the granule also recovered in the peripheral parts of the granule. But the granule, which adsorbed above 18.4 mg. iodine per 100 mg. of starch, could not recovered the original X-ray B-pattern by the addition of thiosulfate solution and gelatinized completely. From these results, it was suggested that the inner part of a potato starch granule might have an amorphous amylose component in higher concentration than the peripheral part of the granule.

On the Denaturation of Crystallin β-Lactoglobulin by the Radiation of Ultraviolet Light. Part I. (p. *667~670*)

By Sadao SAKAMURA and Yataro OBATA

Crystalline β-lactoglobulin isolated from pooled milk according to the method of Aschaffenburg was exposed to UV-light and the resulting denaturation of the protein was characterized on some opysico-chemical properties:

(1) When exposed in aqueous solution containing salt and acetate buffer (pH 4.7) solution, the precipitates of the protein occure in the both cases, but a brown discoloration takes place without precipitation in pH 6.7 of the phosphate buffer.

(2) The electrophoretic patterns of the protein in certain buffers increase the number of the peak on the irradiation and it suggests that an action of UV-light is capable of making the protein inhomogeneous.

(3) The other significant changes are also observed in increasing the absorption at ultraviolet region of spectrum, in flatenning the peak in the ultracentrifuge pattern and in the liberation of ammonia. The last one may be regarded as an factor making the protein electrophoretically inhomogeneous.

Studies on the Quantitative Determination of Sugars in Fruits. Part I. Effects of Conditions of Hydrolysis on the Quantitative Determination of Sucrose in Fruits. (p. *671~673*)

By Kazuo OSODO, Hidejiro KAZUMI, Masayuki KOTAKA and Hanao SHITOMI

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In order to determine the amount of sucrose in fruit juices precisely, the present authors reexamined the conditions of hydrolysis of sucrose which have been reported before, and studied the stability of fructose in the hydrolysis process. It was found that the results which are indicated in the following table were suitable for the determination of sucrose.

Sucrose in Solution	HCl(%)	Temp.°(°C)	Reaction Time (Hour)
about 0.2	0.1	98	1/3
0.2	2.4	30	8
0.2	2.4	40	4
0.2	2.4	50	3/4

Free fructose in the sugar solution was more stable than the fructose which was produced by inversion of sucrose, namely, the latter was decomposed by heating at 50°C for 2 hours in 2.4% hydrochloric acid, but the former did not decompose for 4 hours at the same condition. Therefore, the sucrose in fruits like apple and pear which contain 60-70% free fructose on total sugar could be determined precisely under above-mentioned conditions.

From these results it may be considered that the fructose which is produced by inversion of sucrose has unstable fructo-franose ring and is decomposed into hydroxymethylfurfural immediately.

Hydroxylation of Progesterone by *Aspergillus*.

(Studies on Steroids and Microorganisms, Part I) (p. *674~679*)

By Hiroshi IIZUKA, Atsushi NAITO and Masako HATTORI

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Oxygenation ability of the progesterone were compared with 473 strains of *Aspergillus* preserved in the Division of Type Culture Collection, Institute of Applied Microbiology, University of Tokyo, and have selected some useful strains. It was thereby found that some strains of black *Aspergillus* and their induced mutants had stronger oxygenation ability. And then optimal conditions of culture were selected with some useful strains.

Mass culture with the optimal conditions indicated that the fungi could be classified into two kinds of group. One group of strains produced only 11α-hydroxyprogesterone (m.p. 168–170° $[\alpha]_D^{20}+169°$(c=0.9 $CHCl_3$)), (*A. Usamii* mut. *shirousamii* IAM B-407, *A. awamori* IAM K-0625) and the other produced both 11α-hydroxyprogesterone and 6β,11α-dihydroxyprogesterone (m.p. 232–234° $[\alpha]_D^{20}+152°$(c=0.97 $CHCl_3$)) (*A. saitoi* IAM R-1216, *A. usamii* U.V. mutant IAM 59-1). However, none of these fungi seemed to produce any other products.

Tank culture of *A. saitoi* IAM R-1216 having the strongest oxygenation ability was found to effect the complete oxygenation of the substrate (progesterone) in within 48 hours.

Butanol Fermentation. Part XXIV. Fermentation of Molasses. (5) Fermentation on the Industrial Scale, and Fermentation with Addition of Ammonia. (p. *679～684*)

By Motoyoshi HONGO, Ken NAGATA

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(1) Using the isolated saccharolytic microorganisms (K5, NH or KD strain) in the acetone-butanol fermentation of 9,000 l blackstrap molasses mash, the yields of solvents were more than 30% (respectively 30.4, 31.6 or 32.1%) on the basis of the sugar in 2 days.

(2) Using the strain KD in the fermentation of 180,000 l blackstrap molasses mash, the yield was 29.8% in 40 hrs.

(3) The yield of solvents from some blackstrap molasses was less than 28%. In the case of this molasses, it was found that the addition of ammonium-sulphate or Ca-superphosphate decreased the solvents yield. So, using ammonium-hydroxide in place of ammonium-sulphate without the addition of Ca-superphosphate in 9,000 l mash, the yield reached 30–31% in 40 hrs.

Butanol Fermentation. Part XXV. Fermentation of the Mixture of Molasses and Starchy Waste Cake. (p. *684～689*)

By Motoyoshi HONGO and Ken NAGATA

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(1) In the acetone-butanol fermentation of the mixture of high test molasses and starchy waste cake from sweet potato starch production by the saccharolytic strain KD or L12, it was possible to mix such amounts of starchy waste cake as correspond to 2/3 sugar of total (5–6 g/100ml) in mash. Gas evolution began earlier and the ratio of solvents tended to be butanol rich. In the use of more amounts of starchy waste cake, it was better to use *Clostridium kainantoi* in place of these strains.

(2) Using blackstrap molasses, it was impossible to mix such amounts of starchy waste cake as correspond to more than 1/5 sugar of total in mash. The use of more amounts of starchy waste cake tended to make fermentation abnormal. The partial substitution of high test molasses for blackstrap molasses eliminated this tendency.

(3) In the use of blackstrap molasses, it was possible to mix such amounts of starchy waste cake as correspond to 1/2 sugar of total in mash by use of mixed culture of both saccharolytic strain (KD) and Weizmann type strain (*Cl. acetobutylicum* 314 or *Cl. kainantoi*).

Chemical Studies on the Autolysis of of Meats. Part IV. On the Extraction and Quantitative Determination of Myosin and Actomyosin. (p. *689～695*)

By Masao FUJIMAKI

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The tenderness of meats during aging was found not to be dependent upon the proteolysis of meats, as non-protein nitrogen was not shown to increase during the process except immediately after rigor mortis.

Methods of quantitative determination of myosin, actin, actomyosin, sarcoplasmic protein and stroma were discussed and examined. Revised method of Hasselbach and Schneider was proposed.

Chemical Studies on the Autolysis of Meats. Part V. On the Changes of Fibrillar and Sarcoplasmic Proteins during Aging of Meats.

(p. *695~700*)

By Masao FUJIMAKI and Yutaka NAKAJIMA

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Changes of myosin, actin, actomyosin, globular protein and stroma during aging of veal and rabbit muscles were experimented. With onset of rigor mortis, myosin and actin decreased rapidly, and actomyosin occured and increased. But off rigor and tenderness of meats were not shown to be dependent upon dissociation of actomyosin and also upon proteolysis of meats.

With rigor mortis stroma increased, and with off rigor it decreased. The alterations of actomyosin–S and actomyosin–L (named by authors) seemed to have some relation with rigor mortis and off rigor.

In meats immediatly after death, the content of fibrillar protein was almost same among many kinds of animals, but that of globular protein was different with kinds of animals. Meats with more quantity of globular protein seemed to have more water–binding capacity.

From the results of electrophoresis, water soluble proteins were observed to reveal little changes during aging of meats.

Hiochic Acid, a New Growth Factor for Hiochi Bacteria. Part I. Distribution of Hiochic Acid.

(p. *701~706*)

By Gakuzo TAMURA and Atsuo NAGURA

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Evidence is presented for the existence of a new factor required absolutely for the growth of 'True Hiochi Bacteria', i, e., *Lact. homohiochii* and *Lact. heterohiochii*.

The Sake (Japanese rice wine) which is frequently spoiled by these bacteria contains this factor. It was not detected in liver extract, yeast extract, malt extract, tomato juice, whey, and laboratory peptones, but was found to be accumulated in the culture broth of several microorganisms, such as *Aspergillus*, *Penicillium*, *Monilia*, *Willia*, *Saccharomyces* and *Lactobacillus*.

The factor is an organic acid since it is extracted with ethylacetate or chloroform from the culture broth at acidic pH. It is relatively stable to alkali, but unstable to acid. All of the compounds of known structure examined were completely inactive.

Hiochic Acid, a New Growth Factor for Hiochi Bacteria. Part II. Isolation of Hiochic Acid.

(p. *707~713*)

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By means of bioautography it was confirmed that the new growth factors for 'True Hiochi Bacteria' produced by several organisms in the broth were all the same.

From the broth in which *Aspergillus oryzae* strain A was grown, the factor was extracted with butanol and or ethylacetate after being acidified to pH 2.0. A highly purified syrupy material was isolated by the chromatographic technique using silica gel, and a powdered barium salt of factor was obtained.

When the barium salt was acetylated with acetylchloride or acetic anhydride, its activity for the growth of Hiochi bacteria disappeared completely. However, the activity was recovered after alkali–hydrolysis of the acetyl derivative, indicating the presence of hydroxyl group. No colour was developed upon the addition of ferric chloride. The aldehyde and ketone group tests were all negative. The olefinic unsaturation tests were also negative. It is concluded, therefore, that the factor is a saturated hydroxy acid.

The barium salt of the factor supports the maximum growth of *Lact. heterohiochii* strain H-1 at the concentration of 0.4 μg per ml of the medium. It is proposed to designate the factor by the name 'Hiochic acid'.

Studies on *Strephtomycetes*. Humidin, an Antifungal Antibiotic produced by *Streptomyces humidus*.

(p. *713~716*)

By Kôiti NAKAZAWA, Motoo SHIBATA, Hiroichi YAMAMOTO, Toshihiko KANZAKI, Eiji HIGASHIDE (*Institute for Fermentation, Osaka*), Akira MIYAKE and Satoshi HORII

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Streptomyces humidus isolated from soil by the authors produces dihydrostreptomycin directly in the culture filtrate. The organism also accumulates a new antifungal and antiprotozoal antibiotic, humidin, in the culture mycelium simultaneously. Humidin is extracted with acetone and purified by distribution between ethylacetate and water at various pH's. Characteristics of crystalline humidin are as follows: colorless plates, m.p. 145–146°; elementary analysis, C 63.51,6 3.42, 63.36, H 8.66, 8.52, 8.32, 8.31; molecular weight, 550±50 (Barger), 823±10 (the single crystal X-ray diffraction method); U.V-absorption maximum, 245, 285 mμ; gives orange-red to orange-violet with conc. H_2SO_4; decolorizes $KMnO_4$ and Br_2; negative to Fehlings and $FeCl_3$ reactions. Humidin is soluble in acetone, dioxane, ethylacetate, hot ethanol, *n*-butanol, ethyl ether, methanol, benzene, water, petroleum ether and carbontetrachloride, the solubility decreasing in this order, but almost insoluble in water and the solvents following water. It is active against certain fungi and protozoa, e.g. the minium concentration for complete inhibition of the growth of *Sacch. cerevisiae*, *Torula rubra*, *Ustilago zeae*, *Pythium de' Baryanum*, *Gloeosporium laeticolor* and *Tricomonas vaginalis* are 1 mcg/ml or less when tested by agar dilution method, but 100 mcg/ml or more to *Asp. niger*, *Candida* sp. and *Trichophyton* sp., and inactive against bacteria. The toxicity to mice is relatively strong, e.g. $LD_{50}=4.5$mg/kg intraperitoneally and $LD_{50}=54$mg/kg orally.

A Photoelectric Method of Determination of Total Calcium in Serum and Urine (p. *717~719*)

By Yukio ICHINOSE

(*The National Institute of Nutrition*)

Colorimetric method of determination of total calcium in body fluid has been improved mainly by Joseph H. Roe & Bernard S. Kahn, T. Kuttner & H.R. Cohen.

But this method yet has some weakness in accuracy. Auther found that the mass of $MgNH_4PO_4$ co-precipitated with calcium phosphate in Ca and Mg levels of human serum was analytically significant.

So that he tried as follows:

(1) At the first step, Mg ion was caught by addition of conc. NaOH solution——it was fixed as $Mg(OH)_2$ precipitate.

(2) For the no loss of calcium phosphate through filtration process, from sampling to photoelectric measurement, the operation was tried in same spitz-glass-tube.

Then, accurated results were gained.

Studies on Pungent Principles of Capsicum. Part II. On Constants and Properties of the Two Pungent Principles (p. *720~722*)

By Sadayoshi KOSUGE, Yukio INAGAKI and Minori NIWA

(*Faculty of Agriculture, Gifu University, Gifu*)

The constants and physical-chemical properties of new two pungent principles isolated from Japanese capsicum were studies:

1) Pungency. Both of them are pungent.

2) Melting point. One(I) gives 65.9~66.3° and the other(II) 65.6~65.8°.

3) Elementary analyse. (I) C 70.17%, H 8.60%. (II) C 70.20%, H 8.47%.

4) Molecular weight. (I) 302, (II) 315. (Rast's method)

5) Paper chromatography. (I) gives R_F 0.68 and (II) R_F 0.57, the mixture of 0.1 M-NaOH and 0.05 M-Na_2CO_3 (1:1) being used as the developer. (I) gives R_F 0.47 and (II) R_F 0.22, 0.4 N-HCl saturated with bezene being used as the developer. In both cases Toyo filter paper No. 50 was used.

6) Color reaction. Both give color reaction with many phenol reagents and green color with ammonium vanadate.

7) Ultra-violet light absorption. Both show absorption-curve of the similar form and maximum absorption at 281 mμ in alcohol).

8) Infra-red spectrum (KBr tablet). (I) shows band (inter alia) at 968 cm^{-1} and (II) no.

Hydroxylation of Progesterone by *Syncephalastrum* sp. (Studies on the Hydroxylation of Steroid by Microorganisms. Part I) (p. *723~728*)

By Toshinobu ASAI, Kô AIDA, Eiji OHKI, Tokuji TANAKA and Masako HATTORI

(*The Institute of Applied Microbiology, University of Tokyo*)

The oxidative ability of progesterone was examined with 284 strains of *Rhizopus* sp. Among these, 21 strains were found to have such ability. Mass cultures of five of these strains were carried out and the oxidation products were identified as 11α-hydroxyprogesterone. The yield obtained was 50%, using *R. chinensis* Saito and *R. nigricans* Yamazaki. *R. kansho* Yamamoto was found to oxidize progesterone to 11α-hydroxy- and 6β,11α-dihydroxyprogesterone, the yields of which were 40% and 10%, respectively.

Two kinds of new hydroxy-derivative of progesterone were found to be formed by *Syncephalastrum* sp.

One(I) of them melted at 222-226°; $[\alpha]_D^{18}+122°$ (C=0.9, $CHCl_3$); λ_{max}^{MeOH} 240.2 mμ (ε=17,000). *Anal.*, Found: C, 72.56; H, 8.65. Calcd. for $C_{21}H_{30}O_4$: C, 72.80; H, 8.74%. This is 7α,15β-dihydroxyprogesterone.

The other compound(II) was confirmed to be trihydroxyprogesterone, m.p., 263-265° (dec.) $[\alpha]_D^{20}+170°$ (C=1.1, MeOH); λ_{max}^{MeOH} 240.2 mμ (ε=12,700). *Anal.*, Found: C, 69.59; H, 8.44. Calcd. for $C_{21}H_{30}O_5$: C, 69.58; H, 8.34%.

In order to increase the rate of hydroxylation, examination were carried out on various cultural conditions. (I) was formed in a short time of incubation, while (II) required a long period.

On Available Lysine in Milk Proteins. (1) The Colorimetric Determination of Available Lysine in Milk. (p. *728~732*)

By Susumu ADACHI and Takeo NAKANISHI
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The principles of this method depends upon the reaction of 2,4-dinitro-1-fluorobenzene with ε-NH_2 of lysine in milk. A 0.5 ml sample of milk is added into the test tube containing 100 mg of $NaHCO_3$. Two ml of 2.5 per cent (volume) dinitrofluorobenzene alcohol solution is added into the mixture and shaked for 2 hours in dark place. Then, the mixture is inserted in the boiling water until alcohol is evaporated off. After the addition of 5 ml of 6-N hydrochloric acid in the residual solution, the test tube is sealed and heated at a temperature of 105°C for 15 hours. The hydrolysed solution is filtered and washed completely with water. Two ml of the filtrate that is filled up to 10 ml is extracted three times with 10 ml of ether. One ml of the water solution is adjusted to pH 4.5 and filled up to 10 ml with 5 per cent acetic acid, N sodium hydroxide and water. The absorption coefficient of this solution in 10 ml cell is read by means of electric photometer with K-6 filter ($\lambda_{max}=455m\mu$) using the blank sample subjected to the same treatment except the dinitrophenylation as control solution. The content of available lysine is calculated from the comparison the reading with standard curve of ε-DNP-lysine.

Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
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Paperelectrophoretic Studies on Whey Proteins. (p. *733～735*)

By Rinjirô SASAKI and Umeo YOSHINO

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

Paperelectrophoresis was applied for separation of whey proteins and hydrolysis products of casein by rennin.

The fractionated whey proteins on the strip of filter paper were detected by staining the paper strip with bromophenol blue and the amount of dye combined by separated proteins on paper was measured by dye elution method or direct scanning method. The elution or direct scanned curves were compared with the pattern obtained with the moving boundary method and gave good agreement. β-Lactoglobulin and α-lactalbumin were separated satisfactorily but globulins were not fractionated clearly each other.

Whey from mastitis milk was studied by paperelectrophoresis and a fraction which migrated towards anode faster than β-lactoglobulin peak was detected. This fraction is thought to be serum albumin which usually exists in normal milk in small quantity. In mastitis milk, a kind of abnormal milk, it increased in amount and was detected on paper.

Casein solution was hydrolysed with crystalline rennin and an intact casein was precipitated by adding trichloracetic acid to reaction mixture. 2% trichloracetic acid soluble fraction was concentrated in vacuo and examined by paperelectrophoresis at pH 7.2 with phosphate buffer. Two fraction were observed on paper. Each fraction was extracted with hot water and hydrolysed by hydrochloric acid. The results of the amino acid analysis by paper chromatography showed that the fraction which migrated to anode faster contained more aspartic acid than the other.

Studies on the Hydrolytic Action of Rennin on Casein (p. *736～738*)

By Umeo YOSHINO

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The proteolytic action of rennin on casein was studied. Crystalline rennin hydrolysed more casein than commercial rennet (Hansen's rennet tablet) at pH 6.0. Commercial rennet was thought to contain pepsin because it produced more hydrolysis products than crystalline rennin at pH 2.0.

The results of paper chromatography on 12% trichloracetic acid soluble fraction produced from casein by rennin action showed that no free amino acid was liberated.

2% Trichloracetic acid soluble fraction produced from casein by rennin action was hydrolysed with hydrochloric acid and was analysed for amino acid by paper chromatography. The results showed that all kinds of the amino acids except tyrosine which were contained in casein were detected in the soluble fraction. Absence of tyrosine in soluble fraction was also shown by tyrosine determination with phenol reagent, that is, when nitrogen of soluble fraction was amounted to 6% of total nitrogen, tyrosine in soluble fraction was equal to only 0.8% of total tyrosine in casein. By the action of trypsin (at pH 8.0), papain (at pH 7.5) or pepsin (at pH 2.0) the % of nitrogen and tyrosine moved to soluble fraction were parallel. Pepsin at pH 6.0, however, produced soluble fraction in which little tyrosine was contained.

In 2% trichloracetic acid soluble fraction after ninety minutes' rennin action the increase of the relative amount of aspartic acid compared to that after fifteen minutes' when milk had coagulated was observed.

On the New Antibiotics "Ophiobolin", produced by *Ophiobolus Miyabeanus*. (p. *739～744*)

By Michikazu NAKAMURA and Keijiro ISHIBASHI

(*Takamine Laboratory, Sankyo Co., Ltd.*)

In the course of a search for new and useful antibiotics produced by plant pathogenic fungi, a strain of *Ophiobolus Miyabeanus* was found to produce a new antifungal and antiprotozoal antibiotic, named 'Ophiobolin'.

Ophiobolin is produced in yield of approximately 130 mcg/ml in a medium containing 2% sucrose, 0.5 % peptone, 0.5% KH_2PO_4, 0.25% $MgSO_4.7H_2O$ (pH 5.0), which is incubated with the fungus at 27° for about 40 hours by shaking or tank culture method.

Ophiobolin is easily extracted from the cultured broth with benzene, and the colourless stick-like crystal is obtained from ether solution, m.p. 181°; $[\alpha]_D^{22}=$ 304.5 (c=5.3, chloroform); *anal.* Found: C 74.96; H 8.33. Calcd. for $C_{24}H_{32}O_4$: C 75.00; H 8.33%. $\lambda_{\max}^{n\mu}$ 237.5; $\lambda_{\max}^{\text{Nujol}}$ 2.84μ (-OH), 5.76μ (CO), 5.89μ (ketone type CO), 6.17μ (C=C), 8.06μ (-C-O-C=), 9.37μ (>CH-OH).

The crystal is readily soluble in chloroform, moderately soluble in benzene, ethanol, methanol, aceton, ethylacetate and ether, least soluble in petroleum-ether and water. Stable at pH 2.0 in alcoholic solution but very unstable in alkali.

Ophiobolin is specifically active against *Trichomonas vaginalis*, Trichophytons and some other plant pathogenic fungi (e.g. *Glomerella*, *Gleosporium*) at 1-5 mcg/ml concentrations. The LD50 dose for mice is 238 mg/Kg subcutaneously, 21 mg/Kg intraperitoneally, 12 mg/Kg intravenously and 73 mg/Kg orally.

Ophiobolin inhibits at low concentrations the germinatian of rice seedlings and the growth of roots, and causes a pathogenic symptom of *Ophiobolus Miyabeanus* on rice plant, and a chemically and biologically similar fraction to this substance is extracted from a naturally diseased rice plant.

As the results, it would be considered that the Ophiobolin may be a toxin of *Ophiobolus Miyabeanus* against rice plant.

Non-microbial Deterioration of Preserved Egg Albumen during the Storage. Part III. Coagulation of Albumen and Decomposition of its Sulfur-containing Amino Acids during Dipping the Shell Eggs in Alkaline Solution. (p. *745~749*)

By Yoshinobu NONAMI

(*Faculty of Agriculture, University of Niigata*)

To cause coagulation of albumen, chicken and duck eggs were dipped in alkaline solution (NaCl, IO; $Na_2 \cdot CO_3$, IO; CaO, IO; NaOH, 5%) and preserved at 38°C or room temperature for 4-6 days. Crude ash, methionine and cystine of albumen of these eggs were determined every day during the storage in the alkaline solution. Blended fresh and preserved chicken egg albumen and fresh duck egg albumen were coagulated in test tubes by addition of the alkaline solution and observations were made to get available information for comparison of properties of these eggs. By excess alkalies, coagulated albumen was dissolved and in this case the nitroprusside reaction for SH became positive in the albumen. The amount of alkalies suitable for coagulation of albumen of fresh chicken egg, which was estimated by determination of crude ash of the albumen, was more than that of suitable for coagulation of preserved chicken egg or fresh duck egg. The decomposition of cystine in albumen during the storage in alkaline solution was more significant than that of methionine.

The Relation of Maturity to the Composition of the Seed of *Vicia faba*. Part III. The Amylose Content of Starch. (p. *749~751*)

By Sin'itirô KAWAMURA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kagawa University*)

Starches from immature and mature seeds of *Vicia faba* (broad bean) were fractionated by the use of *n*-butanol following complete gelatinization with 2% sodium hydroxide. The iodine coloration and iodine affinity (by potentiometric titration) were measured on starch, amylose, and amylopectin from immature and mature seeds. Calculation from the iodine affinity showed that the amylose content of starch was 16% for immature seeds and 27% for mature seeds. As the seeds matured, there were observed simultaneous increases in the starch content of seeds, the size of starch granules, the amylose content of starch, and the average molecular weight of starch and amylopectin (from intrinsic viscosity and alkali lability number). These findings suggest appositive growth of starch granules. (See *Chemical Abstracts*, **51**, 15710*ab* (1957) for Parts I and II.)

Studies on the Effect of Homogenization on the Properties of Milk Caseins (p. *752~756*)

By Rinjirô SASAKI and Masahiro IWAIDA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University*)

It was already investigated by means of electron microscope[1)] that the spherical form of casein particles was damaged when milk was processed under homogenization procedure. The authors want to analyze more clearly of the changes of the properties of casein caused by homogenization.

Fresh unpasteurized mixed herd milk was homogenized at 0, 1500+500 and 2500+500 lb p.s.i. (two stage) in a Cherry-Burrel homogenizer. Purified whole casein was prepared from these three divisions of casein by acidification, each of which was dissolved in sodium hydroxide to give a 3% solution of pH 6.8, and was submitted for the measurements of viscosity, tyrosine value, formol titration value and sulfhydryl group. The data indicate that a pronounced decrease (about 50%) occurs in the viscosity of homogenized casein, while the values of tyrosine, formol titration and sulfhydryl group increase a little. Peptic digestion was carried out with hydrogen chloride solutions of the above mentioned three divisions of casein. It is commonly known that in the peptic digestion of the whole casein there appears a viscosity peak, which is attributed to the coagulation of casein particles[2]. Regarding the homogenized milk casein, the peak is a little delayed and is much lower than the unhomogenized. No remarkable change is observed in the electrophoretic patterns.

(1) H. Hostettler and K. Imhof: *Landwirtschaftliches Jahrbuch der Schweiz*, **66**, 309 (1952)

(2) H. Holter, K. Linderstrøm-Lang and J. Funder: *Compt. rend trev. lab.Carlsberg*, **19**, 10, (1932)

Studies on the Formation Mechanism of Black Tea Aroma. Part IV. Effect of the Condition of Black Tea Manufacture on the Formation of Volatile Carbonyl Compounds. (p. *757～758*)

By Toshio NAKABAYASHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University*)

In this experiments, the effect of the condition of black tea manufacture on the formation of volatile carbonyl compounds (VCC) was studied.

On the course of manufacture, VCC changed little in the withering but increased considerably in the rolling and fermentation and this change of VCC resembled to the oxidation process of tannin.

When changed the manufacture condition, the longer the withering was, the more VCC was produced and when the time was long and temperature was high in the fermentation and drying, VCC increased.

It was, therefore, supposed that VCC was produced by the action of the oxidized tannin from some precursor which was produced in the withering.

Butanol Fermentation. Part XXVII. Fermentation in High Concentration of Sugar. (2) Sorption Method by Use of Active Carbon. (1) Sorption of Each Solvent by Active Carbon. (p. *758～763*)

By Izue YAMASAKI, Motoyoshi HONGO and Kyosuke AKABOSHI.

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

Among various carbons, only the active carbon was effective. Sorption on isotherm of acetone or butanol by some active carbon was respectively $x/m=0.151C^{0.52}$ or $x/m=0.275C^{0.57}$. These isotherms showed that butanol was more sorped than acetone in this carbon. The active carbon (6 g/100ml) in the fermented mash, to which acetone and butanol were added as corresponding to ferment 12% sugar mash, sorped 20% of total amounts of acetone and 50% of butanol in 48 hours. The amounts of butanol sorped in carbon were 4 or more times that of acetone. As the concentration of acetone and butanol was higher, this selective sorption was more marked. But butanol–sorption needed more hours than acetone–sorption. Smaller granules of carbon were more effective, but the fine powder was not so good. The smallest granules sorped 67% of total amounts of butanol. Carbon packed in a bag suspended in the fermented mash was more effective than free carbon.

Butanol Fermentation. Part XXVIII. Fermentation in High Concentration of Sugar. (2) Sorption Method by Use of Active Carbon. (2) Fermentation with Addition of Active Carbon. (p. *764～770*)

By Izue YAMASAKI and Motoyoshi HONGO

(*Department of Agricultural Chemistry, Faculty of Agriculture, Kyushu University*)

(1) Concentration of sugar in mash: Fermenting velocity showed that the sugar concentration of 12～18 g/100ml, especially 15 g/100ml, was good. But the weak culture gave somewhat less yield of solvent in the mash of high concentration of sugar. So 12 g/100ml sugar mash was used.

(2) Amounts of active carbon: This was given by the equation [(concentration of produced butanol)–(maximum concentration of butanol tolerated by used culture)]/(amounts of butanol sorped in unit weight of used carbon). When the culture was normal and the amounts of butanol sorped in 1 g of used carbon were about 0.2 g, 5～6 g/100ml of carbon was adequate to ferment 12 g/100ml sugar mash.

(3) Time of addition of carbon to fermenting mash: Its addition after growth phase or maximum acidity phase gave the best results.

(4) Size of carbon granules: 2～4 mm^3 gave good results.

(5) Two phases in the fermentation: In the case of the addition active carbon, increase of fermenting velocity was striking in the mash of high concentration of sugar. It was ascertained that this increase was caused to the high concentration of sugar in early

period and to the sorption of butanol by carbon in later period.

(6) Solvent yield: 12 g/100ml Sugar mash added active carbon (6 g/100ml) was fermented in 3 days by *Clostridium acetobutylicum*. The yield of solvent was 36% (to added sugar). Acetone: butanol was 1:2.

Chemical Studies on the Autolysis of Meats. Part VI. On ATPase Activities of Myosin and Actomyosin in Meats. (p. *770～774*)

By Masao FUJIMAKI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

Conditions for estimation of ATPase activities were discussed. Optimum pH value was found to be 6.8 for myosin–ATPase, 6.5 for actomyosin–S–ATPase and 5.9–6.2 for actomyosin–L–ATPase.

The inactivation of ATPase activity by Mg ion was much more in actomyosin than in myosin.

Activities of ATPase decreased in order of myosin, actomyosin–L and actomyosin–S from same muscles.

During storage of myosin and actomyosin solutions at about 0° for 2 weeks, there were little variations in ATPase activities, but much decrease in sulfhydryl contents. So there seemed to be no parallelism between ATPase activities and sulfhydryl contents of myosin, actomyosin.

ATPase activities of water and potassium chloride extractive solutions of meats decreased during the aging, and this seemed not only due to quantitative changes of myosin and actomyosin in the solution, but to alterations of ATPase activities of these proteins.

Chemical Studies on the Autolysis of Meats. Part VIII. On the Influence of Aging of Meats upon Hydration of Meat Proteins. (p. *775～778*)

By Masao FUJIMAKI and Hiroko KURABAYASHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University*)

Water–binding capacity (considered to reveal degree of protein hydration) of several meats (beef, pork, horse meat, rabbit, chicken and fish) was estimated.

The lowest capacity was found in the pH values of 5.3–5.5.

Addition of each kind of phosphates (ortho–, pyro–, tripoly–, hexameta–phosphates) to meat was found to increase the capacity.

Changes of water–binding capacity during aging of meats were found due to not only variations of pH values of meats, but to reactions of protein which might perhaps occur during the process.

Hiochic Acid, a New Growth Factor for Hiochi Bacteria. Part III. Nutritional Requirements of Hiochi Bacteria. (p. *778～783*)

By Gakuzo TAMURA and Yahiko SUZUKI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

Using a synthetic medium supplemented with hiochic acid as the basal medium, the nutritional requirements of 'True Hiochi、Bacteria', *Lact. heterohiochii* strains H–1, H–3, H–5, and H–11, were studied.

Vitamin omission test revealed that thiamin, riboflavin, nicotinic acid, pantothenic acid and folic acid are needed for the growth of the test organisms. Hiochi Bacteria can not grow in the media from which either Tween–80 or peptides from liver autorisate or casein entimatic digest were omitted. Purine and pyrimidine bases have a growth stimuratory effect.

It is also confirmed that the growth rate of Hiochi Bacteria depends upon the quantity of hiochic acid which are supplemented to the medium.

Hiochic Acid, a New Growth Factor for Hiochi Bacteria. Part IV. Determination of Structure of Hiochic Acid. (p. *783～790*)

By Gakuzo TAMURA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University*)

Hiochic acid was isolated in crystalline forms as quinine salt ($C_{26}H_{36}O_6N_2$, m.p. 137–8°C) or S–benzylthiuronium salt ($C_{14}H_{22}O_2N_2S$, m.p. 122–3°C). A distilled syrupy lactone of this acid was also obtained ($C_6H_{10}O_3$, b.p. 130–135°C/2mm, b.p. 145–150°C/5mm, $N^{19}=1.474$, $[\alpha]_D^{20}-19.9°$, pK$=$4.3a). From the chemical reactions, it was evidenced that the acid was a β–hydroxy acid and had no adjacent hydroxyl group. The infrared spectra of the lactone showed clearly the presence of δ–lactone. Therefore, the lacton of hiochic acid is a saturated β–hydroxy–δ–lactone having a formula of $C_6H_{10}O_3$. The following four structures, β–hydroxy–δ–caprolactone (I), β–hydroxy–γ–methyl–δ–valerolactone (II), β–hydroxy–β–methyl–δ–valerolactone (III), and β–hydroxy-α–methyl–δ–valerolactone (IV), are compatible with the observation described above. Among these, structure I seemed to be most probable in view of the fact that the iodeform formation test was positive. The α,β–unsaturated acid resulted from natural hiochic acid showed a definite peak of absorption at 222 mμ (ε=9,880), while the synthetic parasorbic acid showed only an end absorption. This fact obviously showed that the former is different from parasorbic acid, therefore, the presumption of structure I for hiochic acid must be excluded.

As the structure III is also compatible with most of the known characteristics of natural hiochic acid lactone, it was synthesized from-β-hydroxy-β-methylglutaric aldehyde by Tischenko reaction. The infrared spectra of the synthesized lactone (III) were entirely identical with natural hiochic acid lactone. The biological activity of the synthetic lactone was one half that of natural lactone. From these facts, it is concluded that the lactone of hiochic acid is identical with structure III and that the laboratory isomer of the lactone is biologically active.

Chemical Studies on the Tanning Properties of Animal Skin. Part IV. On the Reaction between Skin Collagen and Some Polyphenols.

(p. *790~795*)

By Yasushi SATO*

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo.*)

In order to throw light upon the reaction between skin collagen and gallotannin, experiments were carried out on the reaction between skin collagen and such compounds as pyrogallol, resorcinol, catechol or hydroquinone which contain two or more OH groups assumed to be primary reactive group of tannin.

These polyphenols are adsorbed by skin collagen only in the presence of water, and adsorbed amounts of these compounds are decreased a little by addition of such strong acid as HCl. The correlation between the concentration in adsorption equilibrium and adsorbed amount in dilute solution is partly similar to it in the case of benzoic acid and salicylic acid. So it is estimated that this reaction is partly complied with the reaction between the dissociated group of collagen and H^+ produced from two or more OH group of these polyphenols.

However, desorption of adsorbed polyphenols are with ease, and considering from the maximum adsorbed amount, the number of OH group, contained in each polyphenol have a close relation with the total number of -CO·NH- groups and -CO·N- groups in skin collagen. So it is estimated that the reaction is mainly complied with the reaction between OH group of these polyphenol and these group in skin collagen.

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Chemical Studies on the Tanning Properties of Animal Skin. Part V. On the Participation of Peptid Chain on the Combination of Gelatin and Tannic Acid.

(p. *795~800*)

By Yasushi SATO*

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

The influence of benzoic acid, salicylic acid, pyrogallol or tannic acid on -CO·NH- groups in gelatin was investigated from the change of ratio of optical density in 6.1μ and 6.5μ of infrared.

Benzoic acid or salicylic acid do not influence, if any, a little on -CO·NH- groups of gelatin. Pyrogallol, probably influence on -CO·NH- groups of it, but tannic acid certainly influence on it. It seems that reactive group of tannic acid is mainly OH group in that case.

As gelatin is a derived protein from skin collagen, so it is estimated that adsorption of tannic acid to skin collagen is mainly due to the reaction between OH group of tannic acid and peptid group of skin collagen.

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Studies on the Toxic Substance of *Gibberella fujikuroi*.

(p. *800~802*)

By Yukihiko NAKAMURA, Tokuji SHIMOMURA and Jôji ONO

(*Department of Agricultural Chemistry, Faculty of Agriculture, Hokkaido University*)

Gibberella fujikuroi was known to produce plant hormone gibberellin. Numerous studies were carried out concerning on this point. Recently it was reported that this fungus putrefies fodders of domestic animals, accordingly toxic or sometimes fatal to animals ate such fodders. The authors carried out the present study to find the toxic substance produced by the fungus. They obtained malonic acid from the filtrate of shaking culture medium of *Gibberella fujikuroi* by means of carbon adsorption and acetone elution. It was ascertained by paper chromatography, elementary analyses, molecular weight determination, infra-red spectrum, etc. As malonic acid shows $M.L.D._{50}$ 1.5 mg/10 g body weight against mouse, the authors deduced malonic acid was one of the toxic substances produced by *Gibberella fujikuroi*.

Studies on Amino-carbonyl Reaction of Milk Products. Part XX. The Mechanisms of Degradation of Lactose. (2) Isolation and Identification of

D-Tagatose in Highly Heated Milk. (p. *802~805*)

By Susumu ADACHI

(*Laboratory of Animal Products Technology, Faculty of Agriculture, Tohoku University, Sendai*)

A monoketose was isolated from skimmilk heated under the pressure at 15 lb/sq. inch for 12 hours by means of culumn chromatography using ion exchange resin Amberlite-IRA-410 (HSO_3'-type), and was identified as D-tagatose from the results of paper chromatography and the comparison of melting point with authentic sample. On the paper chromatograms of skimmilk heated at intervals 2 to 12 hours, the area and concentration of the spots of lactose and glucose were decreased, but that of galactose was increased with increasing heating. The spots of lactulose and tagatose were formed gradually during heating. It may be most reasonable to assume that lactose is transformed and degradated to succesively to lactulose, galactose and tagatose in heated milk. The possibility of participation of Lobry de Bruyn and Alberda van Ekenstein transformation in the formation of these ketoses was also discussed.

Feeding Yeast from Waste Soap Liquor (p. *805~810*)

By Ken-ichi OTSUKA and Seiji TADA

(*Research Institute of Fermentation, Yamanashi University*)

The authors selected the best strain of *Pichia* sp. out of the so-called film forming yeasts which could grow in glycerol media. The yield of yeast is usually 50-70% of glycerol consumed on a dry weight basis in the media which had been made up by waste soap liquor from alkali-saponification of pig's fet.

Since the soap liquor had high alkalinity, it was neutralized by HCl, and then concentrated until 1/10 volume using open oven. After this procedure the precipitated NaCl was thrown away.

It was demonstrated that the medium should be fortified with Mg ion and phosphate for normal growth of the yeast. And the influences of concentration of NaCl and glycerol, and of nitrogen sources in the medium on yield of the yeast were exermined and discussed.

The authors carried out the cultivation of the yeast using pilot plant (volume of tank, 300 l; volume of medium, 125 l) and obtained dried yeast corresponded to 50% yield of consumed glycerol. Since the yeast preparation had good flavor and taste, it was supposed that the yeast was available for feeding.

Studies on the Classification of *Streptomyces griseus* Group. Part I. Classification by the Morphology (p. *810~816*)

By Yujiro HARADA, Shiro ITAGAKI, Shigeo KUBO and Shiro TANAKA

(*Tokyo Research Laboratory, Kyowa Fermentation Ind. & Co., Ltd.*)

(1) Authors studied the morphology of *Streptomyces griseus* group by means of cellophane culture method1) and electronmicroscope.

(2) According to Dr. Krassilnikov's report2), streptomycin-producing strain has different morphology from *S. griseus* and has globe spores and classified in a new species *Actinomyces globisporus streptomycinii*. But it was reasonable to conclude that streptomycin-producing strain has the same morphology as other strains of *S. griseus* (grisein-producing strain, var. *rhodochrous* OKAMI and other type cultures), and the morphology of spores of *S. griseus* group are almost capsular or cylindrical, not globe.

(3) The morphology of *S. olivaceus*, *S. griseolus*, *S. flavovirens* and *S. scabis* were the almost same as *S. giseus*, and *S. albus* and *S. flavus* were the different.

(4) By author's studies on the fluctuation of morphology in many progeny and mutagenic processes, the morphological specification was considered a very stable key in classification.

(1) G. Glolitti and M. Bertani: *J.B.* 65(3), 281-282 (1953)
(2) N.A. Krassilnikov: Lecture at Peipin (1955)

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